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Contents – Sommaire – Inhaltsverzeichnis

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T measure the disappearance rate of in litro labelled albumin copper blood was won by heart-puncture the erythrocytes were reinjected while the plasma was incubated with isotonic Cu^{64}-sol tion for 20 to 30 minutes at 37 C. A fraction to be used as standard was set aside and the rest injected intravenously into the same rabbit Several blood samples of 1 ml were taken from the carotid artery immediately after injection and t different time intervals thereafter The rabbit was kept in E ipan narcosis. Copper content and radioactivity of the blood well as organ samples were measured. The site of copper binding in plasma was checked by paper electrophoresis.

To determine the turnover rate of coeruloplasmin and of in is labelled albumin in rabbit plasma, Cu^{64} was injected intraperitoneally intravenously or subcutaneously into rabbits. At different times following injection blood was taken by heart puncture and the plasma obtained transfused into sister rabbit. Several blood samples of 1 ml were taken from the carotid artery of the narcotised receptor rabbit at different times and copper content and radioactivity in blood as w ll as in organ samples determined. The site of copper binding in plasma was checked by paper electrophoresis in an LKB electrophoresis apparatus. As TRIS-buffer was found to liberate copper both from albumen binding as from coeruloplasmin and the copper migrates with the EDTA component, Sorensen phosphat buffer 0.03 M pH 8.0 was used instead. The voltage used w 150 V at 6.5–9.5 mA and the running time 16 hours. The strips were stained for protein with amido black. Coeruloplasmin was localized by means of the dianisidine reagent (13)

In some experiments small pieces of liver (about 0.5 g) were repeatedly cut out from this organ of narcotized animals at different times after application of the tracer Bleeding was stopped by local application of thrombokinase The liver tissue was either immediately homogenized in a glass homogenizer cooled with ice water and containing phosphate buffer as used for electrophoresis or immediately frozen and later processed. The supernatant of the homogenate was won by centrifugation t about 7000 g

In the in itro experiments freshly taken mouse liver was cut into small pieces and incubated with rabbit plasma containing traces of Cu^{64} at 38 °C, 10 ml plasma per gram of wet liver tissue were pplied. Incubation was started simultaneously in several vessels containing similar amounts of liver by addition of corresponding amounts of labelled plasma Incubation was stopped in each essel t different time the liver slices were washed with Ringer solution and homogenized. Copper content and radioactivity of the liver tissue of the incubation plasma, the supernatant including the washing solution and sediment were determined.

Results

Plasma copper Table I contains the mean values of blood copper determinations in the rabbits studied

When injecting Cu^{64} intravenously into a rabbit the plasma radioactivity falls in the beginning and levels off with time After the lapse of few hours the activity figures remain constant (fig 1)

Table I

Mean alues and standard deviation of copper concentration in rabbit plasma

Plasma total μg		Plasma direct reacting μg		Erythrocytes μg	
67.6	18.7	20.1	8.4	61 3	23.3
	n = 19		18		7

n = number of animals studied.

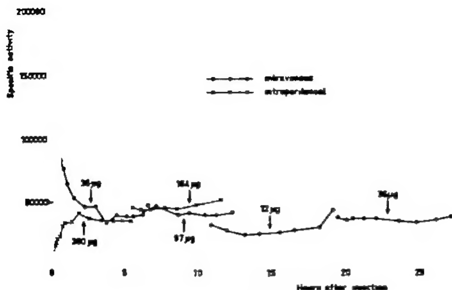


Fig 1 Cu^{64} concentration in blood plasma after intravenous or intraperitoneal injection of radiocopper into rabbits with copper amounts indicated.

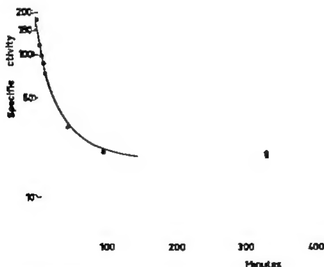
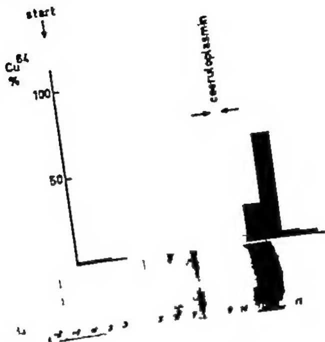


Fig 2. Cu^{64} concentration in the blood plasma of rabbit after intravenous injection of autologous plasma in itro labelled with trace amount of Cu^{64}


start
Cu^{64}
%
100
50
coeruloplasmin


start
Cu^{64}
%
100
coeruloplasmin

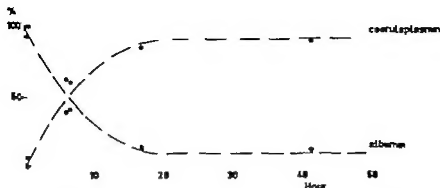


Fig 4 Cu^{64} distribution between coeruloplasmin and albumin of rabbit plasma t different time intervals after intra enous injection of trace amounts of Cu^{64} as deter mined by lectrophoresis.

Two to 4% of the copper administered remain in the plasma independent of the dose and the type of administration (table VI)

If trace amounts of copper are incubated with plasma and the labelled plasma is then intravenously injected into a rabbit, first a rapid fall takes place later the activity remains constant. In four of these experiments about 8% of the radioactivity administered remained in the plasma (fig 2)

Electrophoresis shows that immediately after intravenous injection radiocopper is solely bound to plasma albumin (fig 3a) With increasing time following injection a growing percentage of the radiocopper circulating in plasma is bound to α_2-globulin. By using the dianisidine reagent coeruloplasmin was found to be local ized in the same fraction (fig 3b) Maximal concentration of radiocopper in coeruloplasmin is reached after the lapse of 20 hours. Now 85% of the plasma radiocopper is present as coeruloplasmin copper and the rest as albumin copper Within the time of these experiments, which amounted to 48 hours, this distribution was not changed (fig 4)

When trace amounts of radiocopper are added to rabbit plasma in vitro electrophoretic observations reveal the bulk of radioactivity to be mainly in the albumin fraction. Reinjecting the labelled plasma no change in the copper binding was observed. When increasing the amount of copper added, more and more is found in other plasma protein fractions (table II) Even in human

Table II

Distribution of increasing amounts of radiocopper added in vitro between different, electrophoretically separated protein fractions of rabbit plasma. On the top of the table percentage protein distribution is given. The following sections contain percentage radiocopper bound to protein fractions (with increasing amounts of copper added) as well as the factor F which is obtained by dividing copper percentage by protein percentage.

	Albumin		Globulins β	γ
Protein	4.5	6.6	9.7	9.2
0.074 µg Cu	87.0	3.3	3.3	6.4
ml plasma	F 1.17	0.50	0.34	0.70
0.01 µg Cu	81.2	3.1	4.2	11.5
ml plasma	F 1.09	0.47	0.43	1.25
0.07 µg Cu	79.2	3.2	4.8	12.8
ml plasma	F 1.06	0.49	0.49	1.33
1.07 µg Cu	90.0	3.1	2.1	5.2
ml plasma	F 1.21	0.47	0.22	0.57
1.10 µg Cu	80.0	9.4	5.3	5.3
ml plasma	F 1.07	1.42	0.55	0.58
9.45 µg Cu	61.0	17.2	11.5	10.3
ml plasma	F 0.82	2.61	1.19	1.12

Table III

Distribution of radiocopper added in vitro on the different electrophoretically separated protein fractions of human plasma. On the top of the table percentage protein distribution is given. The following section states the percentage of added radiocopper bound to the protein fractions as well as the factor F which is obtained by dividing copper percentage by protein percentage.

	Albumin		Globulins β	
Protein	82.7	7.6	4.5	5.4
1.07 µg Cu	63.2	12.4	12.6	11.7
ml plasma	F 1.31	0.61	0.34	0.45

plasma trace amounts of radiocopper are mainly bound to albumin (table III). One ml of rabbit plasma can bind up to 800 µg Cu as brought out by electrophoresis, when more copper is added denaturation of proteins occurs.

By incubation of rabbit plasma with trace amounts of radiocopper and intravenous reinjection of the labelled plasma, we were able to measure the disappearance halftime of the in vitro labelled albumin. The results of these studies are shown in table IV. The mean half disappearance time of the radiocopper from the albumin fraction amounts to 9 minutes, when the initial portion of the disappearance curve is interpolated. When the amount of copper

Table II

Disappearance half time of radiocopper added to rabbit plasma in vitro after intravenous reinjection.

Incub. time minutes	Amount inj. ml	Cu conc. µg/ml	Cu inj. µg	$T_{1/2}$ min
22	13.0	0.99	12.90	9 0
30	6.5	0.06	0.39	8.5
50	13.0	0.10	1.30	8.5
95	7.5	1.07	8.00	9.5
20	11.5	9 46	109.00	16.0

added to plasma is increased, the half time of disappearance is prolonged. Interpolation of the initial part of the disappearance curve gave in all cases reasonable values for the plasma volume. In a patient with cancer of the uterine cervix (stage III) a disappearance half time of 7 minutes was found, even in this case a reasonable value for the plasma volume was obtained.

By injecting heavy doses of radiocopper into rabbits and waiting for several hours, plasma was obtained which contained mainly radiocopper bound to coeruloplasmin and only a small part bound to albumin (cf. fig 3b) On injection of this plasma into sister rabbits the plasma radioactivity of the accepter decreased according to a two component curve (fig 5) The first and faster component showed some increase in half time value with increasing incorporation time of Cu^{64} in the donor the mean value being about 38 min (table V) interpolation of the curves gave in all cases reasonable values for the plasma volume During this time radiocopper could be demonstrated in the albumin as well as in the coeruloplasmin fraction of the acceptor plasma (cf fig 3b) The second part of the disappearance curve showed a slower linear fall on semilogarithmic plot (cf. fig 5) the mean value from 5 experiments being 34 hours (table V) During this time the only radiocopper found in the plasma of the acceptor was bound to coeruloplasmin (fig 6)

We calculated turnover figures for albumin bound copper and coeruloplasmin copper making use of the mean values for copper concentration (table I) and disappearance half time (38 minutes respectively 34 hours) given and assuming 100 ml as plasma volume. 520 µg albumin copper and 23.5 µg coeruloplasmin copper are calculated to be transported per day coeruloplasmin transport being 4.5 / of albumin transport. As maximally 8 / of the copper injected, bound to albumin, reappear in the circulation, and as

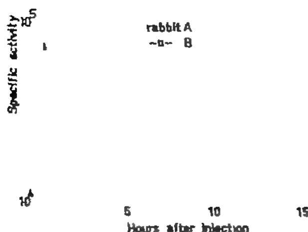


Fig. 5. Cu^{64} concentration in blood plasma of two receptor rabbits at different time intervals after intravenous injection of isologous plasma in vivo labelled for 18... hours in donor rabbit.

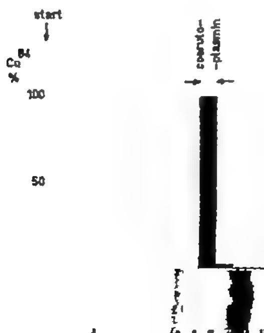


Fig. 6. Cu^{64} distribution on an electrophoresis strip ... on rabbit plasma taken 3.5 hours after intravenous injection of in vi... Cu^{64}-labelled plasma ... rabbit A fig. 5

Table V

Cu^{64} disappearance half time of in vivo labelled rabbit plasma after transfusion into sister rabbits.

Donor			Acceptor	
Application	Incorporation hours	ml plasma transfused	fast component minutes	slow component hours
i. p.	3.5	9 0	19	—
i. p.	18.0	6.0	38	—
i.	6.7	20.0	33	—
i.	16.8	20.0	—	38
i. v	16.8	34.0	—	34
i.	18.5	8.0	41	37
i.	18.5	9.5	41	—
i.	18.5	10.0	—	36
i.	18.5	10.0	—	[illegible]

about 85 / of this amount is coeruloplasmin the percentage of albumin copper directed to coeruloplasmin is calculated to make out 6.8 $/_{o}$, which is in good agreement with the 4 5 / share of the coeruloplasmin turnover in that of the albumin copper turnover When albumin copper turnover is calculated using the disappearance half time of 9 minutes measured after in vitro labelling a figure of 2220 μg Cu per day is obtained.

Organ copper In some of our experiments we determined the Cu^{64} content of the organs of the rabbit at different times after radiocopper injection (table VI) Most of the radioactivity was found in liver guts and muscles. All values, especially those of the blood must be related not only to the time interval elapsed between injection and sampling but also to the type of Cu^{64} labelled compound given. As indicated in table VI we found more copper in the organs and less in the blood when transfusing in vitro than in vivo labelled plasma. After 6 hours of circulation liver activity showed a heavy increase but in this case copper was given in the ionized form. With longer circulation time the radioactivity of the liver decreases, while that of the intestine increases. The gall contained only very small amounts of radioactivity

In table VII the results of determinations of both stable and radioactive copper are stated. Other experiments gave very similar results. The total copper content in the rabbits studied varied between 2.0 and 2.5 mg Most copper is found in the intestine followed by the muscles and the liver Radiocopper concentration is highest in the liver followed by heart, kidneys and intestine. Also

Table VI

Radiocopper content of the organs of the rabbit at different time intervals after application of Cu^{64} in differ forms. Bone marrow assumed to be 1% and muscles to be 50% of body weight.

Application	Solution	Volume ml	μg Cu	Circulation time hrs	Blood %	Liver %	Kidney %	Spleen %	Heart %	Lungs %	Bone marrow %	Muscles %	Intestine %	Urine %
i.v	plasma + Cu^{64} in vitro	11.5	104.2	2.4	9 7	31.0	2.4	0.1	0.3	0.9	0.9	13.7	41 1	0.1
i.	plasma transf. after 6.7 hrs	20.0	33.5	2.7	33.5	24 7	2.7	0.1	0.3	0 9	1.9	11.2	24.6	0.2
i.v	Cu^{64}-solution isotonic	12.9	412	6.7	2.3	33.0	1.3	0.1	0.2	0.5	2.3	7.3	32.5	0 6
i.p	Cu^{64}-solution isotonic	14.0	164	12.0	1.2	17 4	—	0.1	—	—	—	—	—	0.5
i.	Cu^{64}-solution isotonic	10.0	31	40.3	2.1	28.1	7.0	0.1	0 8	1 1	—	12.1	48.2	0.4
s.c.	Cu^{64}-solution isotonic	10 0	21	41.2	2.1	24 1	5.5	0 1	0.7	2 1	—	12.3	52.7	0.2

Table VII

Radiocopper content, chemical copper content, specific activity and specific copper and radiocopper content of rabbit organs. The animal was intravenously injected with 11.5 ml autologous plasma incubated with 104.2 μg Cu containing Cu^{64} and killed after 2.4 hours. The muscle weight was assumed to be 50% and the bone marrow 1% of the body weight. The recovery of the radioactivity was 84%.

	% Cu^{64} recovered	μg Cu total	Spec. act.	μg Cu/g tissue	% act./g tissue
Blood	9.7	83	0.114	0 60	0 060
Liver	31.0	323	0.096	3.61	0.347
Kidneys	2 4	50	0.048	3.27	0.157
Spleen	0.1	1	0.070	1.07	0.050
Heart	0.3	29	0 011	3.44	0.039
Lungs	0 9	26	0.036	2.00	0.072
Bone marrow	0.9	20	0.046	0.33	0.025
Muscles	13.7	800	0 017	1.00	0.014
Intestine	41 1	925	0.044	2.09	0.093
Urin	0.1	20	0.003	0.34	0 001

the specific activity of the organ copper depends on the type of copper compound administered. If copper salt is given intravenously specific activity is highest in bone marrow followed by liver and intestine up to 40 hours after injection. In all our experiments taking more than 2 hours the radioactivity per gram tissue was found to be highest in the liver followed by kidneys. The recovery in these experiments was 99% with a range of 84 to 109%.

Table VIII

Specific activity of rabbit plasma, washed intestine, faeces and gall at different time intervals after intravenous copper injection.

	Plasma cpm/g	Ileum cpm/	Faeces-Ileum cpm/g	Colon cpm/	Faeces-Colon cpm/	Gall cpm/g
11 hours after 212 μg Cu^{64} i.	67382	86320	255711	124846	514067	—
46 hours after 263 μg Cu^{64} i.	33673	70073	27786	51871	155512	162155

In two experiments Cu^{64}-solution was injected intravenously and after 11 respectively 46 hours radioactivity determined beside in the plasma in the intestine and the faeces, in one case even in the gall. The results of these experiments are shown in table VIII. It can be seen that in the short time experiment the contents of the colon as well as that of the ileum was about four times more active than that of the corresponding intestinal wall, while the specific activity of the intestinal wall not much differed from that of the plasma. In the long time experiment the contents of the ileum was about one third as active as the intestinal wall, whereas the Cu^{64} contents of the colon was still about three times higher than that of the gut wall. The gut wall activity was similar to that of plasma. 1 g of gall was about five times as active as 1 g of plasma.

When incubating mouse liver slices with Cu^{64} labelled rabbit plasma in vitro the radiocopper activity of liver tissue was found

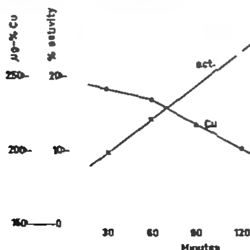


Fig. 7 Change of Cu^{64} content of liver tissue and copper content in plasma with time, when incubating mouse liver slices in Cu^{64} labelled rabbit plasma.

to increase linearly by 8% per hour between 30 and 120 minutes (fig 7) At the same time the copper concentration of the plasma decreased almost linearly Calculating the copper uptake by liver tissue in the three consecutive 30-minute intervals from these data we found 19 18 and 17 µg Cu/hour/g liver tissue. On homogenization of the liver tissue and subsequent electrophoresis no radioactivity was found in the albumin or coeruloplasmin region, it was instead confined to the γβ-globulin region. Centrifugational separation of the homogenate did not reveal a significant difference between supernatant and sediment.

In vivo experiments in rabbits gave similar results (fig 8) Radioactive coeruloplasmin could not be demonstrated in liver homogenate. In the corresponding plasma samples however radioactive coeruloplasmin was present. Our trials to demonstrate coeruloplasmin in liver homogenate by means of the dianisidine method were abortive. When mixing plasma containing labelled coeruloplasmin with inactive liver homogenate the labelled coeruloplasmin did not change its position on electrophoretic separation. This observation excludes the possibility that coeruloplasmin in liver homogenate couples with other substances changing its electrophoretic mobility

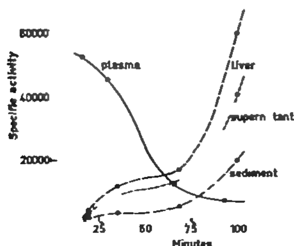


Fig 8. Cu^{64} concentration in plasma, liver tissue, and supernatant and sediment of liver homogenate, after intravenous injection of tracer amount of radiocopper into rabbit.

Discussion

The blood copper values found in our rabbits (table I) are somewhat different from those reported by MARKOWITZ et al. (14) for the human. The total plasma and erythrocyte copper is lower the direct reacting copper is higher than in the human. Similar values were found for the cat (22) and the pig (23)

The rapid fall of plasma radioactivity observed after intra venous injection of radiocopper is partly due to disappearance of ionized copper from the circulation and partly to disappearance of albumin bound copper SCHUBERT AND RIEZLER (5) found thus never more than maximally 60 / of the injected amount immediately after injection in the plasma, 40 % having disappeared in the ionized form. Similar results were reported by COMAR et al. (6) When taking plasma samples some minutes after intravenous radiocopper injection the main part of the copper is found to be bound to the albumin fraction. The radiocopper content of this fraction decreases successively with time, at the same time radiocopper appears in the α_2-globulin fraction, at the same place on the electrophoresis strip at which by means of the dianisidine reagent (13) coeruloplasmin activity is localized. In plasma samples taken between 20 and 48 hours after radiocopper injection about 85% of the plasma copper are found in this plasma protein fraction, which we therefore consider to represent coeruloplasmin. These results correspond well with earlier findings (5 21) We were however not able to reproduce the slight increase in plasma radioactivity after the initial fall observed by WOLFF et al. (21) A possible explanation of this discrepancy may be too sparse sampling in the beginning of the last mentioned experiments (21) Considering the quite high experimental variation, radioactivity distribution between albumin and coeruloplasmin mirrors quite well the percentage distribution between direct reacting and coeruloplasmin copper in samples taken later than 20 hours after Cu^{64} administration. This fact suggests the conclusion that copper contained in both fractions could be in equilibrium with the same copper pool. A similar relation can be deduced from the human data of STERNLIEB et al. (11)

It is of interest that the type of parenteral application of radiocopper as well as the amount given in the range of 5 to 360 μg Cu has no influence on the percentage of the dose remaining in plasma after longer time. The values between 2 and 4% found by us cor

respond well with earlier findings of SCHUBERT (5–7) who found values between 2 and 6% for the human, rat, guinea-pig and dog, WOLFF et al. (21) found 2% for the dog and guinea-pig but 14% for human and rabbit, and STERNLIEB et al. (11) found 20% for the human. The percentage remaining in plasma as found in our studies is about equal to the percentage which plasma copper makes out of the total copper of the rabbit. This suggests the conclusion that the copper injected is mixing quite completely with all the copper contained in the rabbit. This conclusion is further strengthened by the fact that, in the dose range tested by us, the amount given has no influence on the percentage remaining in plasma, which can only be the case when mixing occurs. In contradiction is however the fact that we observed quite high differences in specific activities between different organs. More information on this point could be obtained by applying the longer living Cu^{67} as an indicator.

When rabbit plasma is labelled with trace amounts of Cu^{64} in vitro, copper preferentially binds to albumin, and the same is the case with human plasma. With increasing doses copper shifts over to the other plasma proteins. This finding is in accordance with that of most of the published studies (15, 16–18, 19) but in contrast to those of WOLFF et al. 1/ 21) who however used copper doses in excess of ours. Copper bound to albumin is considered to be the transport form of this metal in the body (3, 9, 15). We found quite a big difference in the disappearance rate of copper bound in vivo and in vitro to albumin. The last mentioned fraction ($T_{1/2}$ = 9 min for the rabbit and 7 min for the human) disappears faster than the first mentioned one ($T_{1/2}$ = 38 min) suggesting a difference in the copper binding. The disappearance rate of transport copper in the in vivo experiments is presumably the physiological one. This last conclusion is further strengthened by the fact that in spite of considerable biological variation turnover values for transport copper calculated using the slower half-time correspond very well with coeruloplasmin turnover values. This should be the case if the anticipated relation between both proteins exists.

Plasma taken from rabbits which received radiocopper more than 20 hours before shows about 80% of its radioactivity in the coeruloplasmin fraction of the plasma. When such plasma is transfused into sister rabbits and the disappearance of the label from the plasma of the receptor is followed, after the labelled albumin has

disappeared, a mean half time of about 34 hours is obtained This is in sharp contrast to the disappearance of the labelled albumin Up to date only values for coeruloplasmin turnover in the mouse and the human are known. For the mouse 20 hours (10) and for the human about 5 days have been found (11)

The results of copper distribution studies confirm the important function of the liver in copper storage as already pointed out by others (6 7) but it is of great interest that a big part of the injected copper is taken up by the muscles. The high radiocopper content of the intestine is caused by the high activity of the faeces. The experiments in which the radioactivity of the intestinal wall was compared with that of the faeces show clearly that copper is eliminated via the intestine (cf 7) Only very little radiocopper was found in urine. In contrast to others we did not find much activity in the gall. We are inclined to interpret our data that excretion in the rabbit takes place directly into the intestine (cf. 7)

The copper concentrations in organs found by us are similar to those found by MARKOWITZ et al. for the human (14) and STERNLIEB et al. for rat liver (27) The uptake of radiocopper by mouse-liver incubated in vitro was found by us to be twice as high as that found by SALTMAN et al (28) for rat liver

We did not succeed in demonstrating labelled coeruloplasmin in the liver It is generally believed that coeruloplasmin is synthesized in this organ (27) Some indications of coeruloplasmin formation in the liver were found by LANG AND RENSCHLER (25) The authors could however not prove that the radioactivity measured by them in the liver was incorporated in coeruloplasmin. As the fraction studied, according to their statement, contained α- and β-globulins, it presumably contained the protein fraction as well which we found to be labelled in our experiments with rabbit liver This fraction migrated as a β-globulin, not being coeruloplasmin however and is possibly identical with one of the copper depot proteins described (26, 29) STERNLIEB et al. (27) mention the appearance of Cu^{64} labelled coeruloplasmin in experiments with perfused rat liver The same authors (27) show that the time course of Cu^{64} specific activity in rat liver microsomes closely mirrors that of coeruloplasmin in plasma. This result is taken as evidence for coeruloplasmin synthesis being confined to liver microsomes, but coeruloplasmin was not identified in the microsomes. We could not observe the decline in the radioactivity of the liver supernatant

reported by the authors. In our experiment about a tenth of the amount of copper used by STERNLIEB et al. (27) was given to an animal being more than ten times heavier Even HOCHWALD et al. (30) could not with certainty demonstrate in in vitro experiments coeruloplasmin synthesis in liver tissue.

These considerations show that indications for coeruloplasmin synthesis going on in liver are up to date only very indirect. It may indeed be very difficult to demonstrate coeruloplasmin synthesis in liver as this organ takes up a big part of radiocopper injected, whereas its coeruloplasmin content (as determined by immunological methods in the human) is only 4.5% of its total copper content (14) Furthermore it is possible that newly synthesized coeruloplasmin leaves the liver shortly after its synthesis. Another possibility is that coeruloplasmin is synthesized in another organ than the liver

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Summary

Blood copper values in rabbits are: plasma total 67.6 μg% plasma direct reacting 0.1 μg%, erythrocyte 61.3 μg%. Trace amounts of Cu^{64} injected into rabbits remain to about 4 in the plasma, 85 of this amount are after 0 hours bound to coeruloplasmin and remain there. Traces of Cu^{64} bind in vivo and in vitro to plasma albumin. The disappearance half time of albumin copper labelled in vivo is after intravenous injection 38 minutes. I vivo labelled coeruloplasmin has half time of 34 hours. Albumin copper turnover relates well with coeruloplasmin copper turnover indicating that copper is transported by albumin. Copper is mainly stored in the liver the muscles contain least half as much copper as the liver The rabbit excretes copper via the intestine substantial excretion via the bile could not be found.

Résumé

Les lapins ont les taux de cuivre sanguins suivants plasma total 67,6 μg%, plasma réagissant directement 0 1 μg% érythrocytes 61,3 μg% Si l'on injecte des traces de Cu^{64} à des lapins, il en reste 4 dans le plasma 85 de cette part sont fixés à la coeruloplasmine 0 heures après et y restent. Des traces de Cu^{64} se fixent in vivo et in vitro à l'albumine du plasma. Après l'injection intraveineuse la période de demi-vie du cuivre fixé à l'albumine et radio-marqué in vivo est de 38 minutes. Le temps de demie vie de la coeruloplasmine marquée in vivo est de 34 heures. Le turnover du cuivre fixé à l'albumine correspond bien turnover du cuivre de la coeruloplasmine ceci démontre que le cuivre est transporté par l'albumine Le cuivre est surtout déposé dans le foie les muscles contiennent à peu près la moitié moins de cuivre que le foie. Le lapin excrète le cuivre par les intestins une excrétion substantielle par la bile pu être démontrée.

Zusammenfassung

Kaninchen haben die folgenden Blutkupferwerte Plasma total 67,6 μg%, Plasma direkt reagierend 20,1 μg% Erythrocyten 61,3 μg% Injiziert man Kaninchen Spuren von Cu^{64} so verbleiben 4% im Plasma, 85% dieser Menge sind nach 20 Stunden an Coeruloplasmin gebunden und verbleiben dort. Spuren von Cu^{64} binden sich in vivo und in vitro an Plasma-Albumin. In vivo markiertes Albuminkupfer verschwindet nach intravenöser Injektion mit einer Halbwertzeit von 38 Minuten. In vivo markiertes Coeruloplasmin hat eine Halbwertzeit von 34 Stunden. Der Albuminkupfer Umsatz stimmt gut mit dem Coeruloplasminkupfer Umsatz überein dies zeigt, dass Kupfer vom Albumin transportiert wird. Kupfer wird hauptsächlich in der Leber gespeichert, die Muskeln enthalten nahezu halb so viel Kupfer wie die Leber Das Kaninchen scheidet Kupfer über den Darm aus, nennenswerte Ausscheidung über die Galle konnte nicht gefunden werden.

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Bone Marrow Lymphocytes of the Rat as Studied by Autoradiography*

By CHARLES G. CRADDOCK

A number of reports have suggested that the small lymphocytes in the bone marrow of rats and guinea pigs have a different rate of turnover from small lymphocytes in the peripheral lymph nodes and spleen (12, 13). These findings have been implemented by the studies of EVERETT et al. (8, 18) who found a high percentage labeling of bone marrow lymphocytes in normal guinea pigs 24 to 48 hours after a single dose of tritiated thymidine (H^3Tdr). The percentage of labeled small lymphocytes increased from near zero a few hours after H^3Tdr to a maximum of about 40% over a 3 day period, following which there was a rapid decline. These findings are in contrast to the small percentage of labeled small lymphocytes which appear in the lymph nodes from the division of larger precursor cells which can incorporate H^3Tdr into DNA. Peripheral lymphatic tissue exhibits a slow turnover (16) and prolonged presence of labeled small lymphocytes.

The following results in normal rats support the view that a major portion of the small lymphocyte population in the bone marrow is self-sustaining, has a turnover similar to that of thymus lymphocytes, and exhibits a low uptake of the isotope into progenitor cells. These findings suggest the similarity of the thymus and bone marrow lymphocyte populations.

Materials and Methods

H^3Tdr 6.4 c/mM (New England Nuclear Corporation) was administered as single subcutaneous injection to normal male Sprague-Dawley rats at 7:30 a.m. Rats were serially sacrificed by exsanguination at intervals indicated in table I. Animals

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shown in table II were given 3 doses of 0,5 μc/g and were sacrificed after the last injection as indicated. Touch preparations of freshly cut sections of thymus and mesenteric lymph nodes were mad on gelatin-coated slides. Marrow preparations were made with able paint brush dipped in bovine serum albumin (5%). The preparations were fixed, layered with Kodak AR 10 stripping film, exposed for 28 days, developed and stained as previously described (6) One thousand small lymphocytes were examined to determine the percentage and intensity of labeling. Background grain counts in these preparations did not exceed 1 grain per 1000 micra and were usually much less than this. Cells with 2 or more grains over the nucleus were counted as labeled. A total of 1000 small lymphocytes in marrow lymph nodes and thymus cortex were counted, 200 cells on each of five preparations. Small lymphocytes in thymus and lymph nodes were, of course, readily identifiable because of the relatively homogenous type of cell present. Cells with nuclear diameter of 6μ or less were counted. I the marrow small lymphocytes are more difficult to identify because of their similarity in size to rubricytes and metarubricytes. Distinction between small lymphocytes with nuclear diameter of 6μ or less was based upon the lighter staining nucleus, scanty cytoplasm often concentrated t one pole of the cell, bsent cytoplasmic hemoglobin and less cytoplasmic and nuclear RNA, as indicated by the degree of pyrinophilia with the methyl-green pyronin stain.

Results

Rats sacrificed one hour after a single dose of H^3Tdr showed a very low percentage of labeled small lymphocytes in the bone marrow lymph nodes or thymus. The large and medium sized progenitor lymphocytes in the lymph nodes showed uptake of the H^3Tdr to an extent comparable to the findings of others (1 9) and have been reported elsewhere (7) Cells of the myeloid and erythroid progenitor series were well labeled (42 / labeled cells, mean grain count 36) Five hours after H^3Tdr a few labeled small lymphocytes were present but the differences between the two periods are slight and probably not significant. At 24 hours there was a large increase in the number of labeled small lymphocytes in the bone marrow due mainly to an increased percentage of cells containing 2—5 grains. A small percentage of the labeled small lymphocytes in the marrow were in the 11—40 grain category The thymus cortex showed an increase in numbers of lowly labeled small cells. The lymph nodes, however showed a very low percentile labeling of small lymphocytes and many of these were in the higher grain count range. At 48 hours the values are essentially unchanged. At 72 hours and 144 hours there was a drop in the percentage of marrow small lymphocytes in the 2—5 grain and 6—10 grain categories. A few highly labeled small lymphocytes remained in the marrow

The fluctuations in the numbers of labeled marrow small lymphocytes were due chiefly to alterations in cells containing only a few grains per cell. The small lymphocyte population in the thymus

Table I

Grain counts of small lymphocytes in bone marrow mesenteric lymph node and thymus cortex in normal rats sacrificed after single dose of H^3Tdr 0.5 $\mu c/g$.

	Time after H^3Tdr	Small lymphocytes unlabeled	Grains per cell 1–5	6–10	11–40	total
	Hours	%	%	%		%
Marrow	1	93.8	4.0	[illegible]	0.2	4.2
Lymph node		99.8	0	0	0.2	0.2
Thymus cortex		100	0	[illegible]	0	0
Marrow	5	98.6	1.0	0	0.4	1 4
Lymph node		98.1	0.2	0.3	0.4	0 9
Thymus cortex		96.8	1 4	2.0	[illegible]	3.4
Marrow	24	61.0	37.6	1 4	1.0	40.0
Lymph node		95.1	1.9	0.9	2.1	4.9
Thymus cortex		57.2	39 4	3.4	0	42.8
Marrow	24	53.0	39 4	4.2	3.4	47.0
Lymph node		95.0	2.8	0.9	1.3	5.0
Thymus cortex		50.5	37.2	12.2	0.1	49.5
Marrow	48	65 6	32.0	0.8	1.6	34 4
Lymph node		90.7	5.2	1 1	3.0	9.3
Thymus cortex		61.0	37.0	2.0	0	39.0
Marrow	[illegible]	85.8	12.0	1.0	1.2	14.2
Lymph node		88.1	5.3	2 0	4.6	11.9
Thymus cortex		79 0	18.5	2.5	0	21.0
Marrow	144	[illegible]	7.0	1.2	3.0	11.2
Lymph node		86.1	1.5	8.6	3.8	13.9
Thymus cortex		93.2	6.4	[illegible]	0.4	[illegible]

Sprague-Dawley male rats, average weight 150 gm.
H^3Tdr 0.5 $\mu c/g$
1000 small lymphocytes counted in each area.

cortex showed a similar increase at 24 hours after H^3Tdr and a subsequent decrease in the percentage of cells containing small amounts of the label. Indeed, cells with more than 10 grains are rarely encountered among the small cell population of the thymus.

Animals receiving H^3Tdr on each of 3 days before sacrifice show a very high percentile labeling of bone marrow small lymphocytes (table II) The majority of labeled cells are in the low grain count categories. These animals showed a steady fall in the number of lowly labeled small lymphocytes but a retention of a small percentage of highly labeled small cells. Indeed, the percentage of cells with more than 20 grains appeared to increase slightly

Table II

Grain counts of small lymphocytes in the bone marrow of normal rats sacrificed after three doses of H^3Tdr 0.5 μc/g at 24 hour intervals.

Time after H^3 Tdr*	Labeled marrow small lymphocytes 3–5	6–10	11–20	21–80	total
Hours			%	%	
1	63.0	11.0	6.9	3.1	84.0
24	56.8	15.0	4.8	5.2	81.8
48	52.0	18.6	3.2	2.6	76.4
72	48.1	22.3	1.2	4.2	75.8
360	16.3	3.2	3.6	4.7	27.8
720	2.3	0.5	4.2	4.8	11.8

* After last of 3 doses of H^3Tdr 0.5 μc/g at 24 hour intervals.

Discussion

The marked increase in the percentile labeling of small lymphocytes in normal rat bone marrow between 1 hour and 24 hours after H^3Tdr suggests that a large percentage of the small lymphocytes in rat bone marrow have a rapid turnover as pointed out by others (8, 18). The similarity of the pattern in the bone marrow to that of the thymus cortex is interesting because both populations of cells appear to have a rapid turnover and both show a low mean grain count. In the normal rat thymus it has been shown that the uptake of H^3Tdr per large lymphocytic progenitor cell is much lower (mean grain count 10.4) than in peripheral lymph nodes (mean grain count 35) after 0.5 μc/g of H^3Tdr (7). The per cent of large and medium sized cells which are labeled 1 to 5 hours after H^3Tdr is at least as high in the thymus cortex as in the lymph nodes. The reasons for this low per cell uptake of the isotope into thymus DNA is not entirely clear but may involve dilution of the labeled thymidine by intracellular pools of thymine nucleotides or thymidine which may be utilized by cells in DNA synthesis (19) or reutilization of DNA or thymidine resulting from DNA breakdown (5). Circulatory and other anatomical factors influencing the availability of H^3Tdr for incorporation by cells in DNA synthesis could also be a factor.

The high mitotic index of the rat thymus cortex (15) and the rapid rate of cell renewal as determined by radioisotopic techniques (2) indicate a more rapid turnover of lymphoid tissue in the thymus than in peripheral lymph nodes. This is further indicated by the high percentage of small thymus cortex lymphocytes which is labeled at 24 hours after a single dose of H^3Tdr. However, these cells contain small amounts of the isotope, as indicated by their low

grain count, because the progenitor cells which divide to produce the small cells had a low grain count, as previously explained. Many large progenitor cells in the lymph nodes, in contrast, show a higher initial uptake of H^3Tdr into DNA and divide to provide small lymphocytes with a higher grain count than in the thymus cortex. However the percentage of labeled small lymphocytes at 24 hours after H^3Tdr is much lower in the lymph nodes than in the thymus, consistent with the slower turnover of the bulk of small lymphocytes in lymph nodes.

The rat bone marrow 24 hours after H^3Tdr contains a few highly labeled small lymphocytes and many small lymphocytes containing 2—5 grains per cell. The small percentage of highly labeled cells does not decline rapidly with time as does the percentage of lowly labeled small lymphocytes. This is particularly evident in the rats which received 3 doses of H^3Tdr at 24 hour intervals. It would appear therefore, that rat bone marrow contains a high percentage of small lymphocytes which exhibit the same kinetics, insofar as H^3Tdr labeling of DNA is concerned, as thymic lymphocytes.

These observations do not settle the question of whether the small lymphocytes which appear to have a rapid turnover in the marrow are manufactured in the bone marrow by replication of lymphocytic progenitor cells or migrate into the bone marrow from the thymus, spleen, or lymph nodes. OSMOND AND EVERETT did not observe sufficient numbers of labeled large or transitional lymphocytes to account for the large numbers of labeled small lymphocytes appearing in guinea pig bone marrow (18) A similar impression was gained in these rat experiments. The percentage of initially labeled immature cells which were lymphocytes was less than 5 / of all labeled cells. However the difficulties in positive identification of very immature progenitor cells as being in one or another cell lineage may have provided an erroneous estimate. OSMOND AND EVERETT on the basis of experiments in guinea pigs with an area of marrow occluded from contact with H^3Tdr favored intramedullary proliferation as the source of this population of small lymphocytes with a rapid turnover (18) Normal rats, given a single dose of H^3Tdr show retention of total H^3DNA in the thymus for 3 days (fig 1) Presumably labeled cells do not move out of the organ for this period of time. If this interpretation is correct, then the high

Normal rats (150—350 gm) Thymus H^3 DNA (●) and H^3 content (△) after H^3 Tdr 0.5 uc/gm

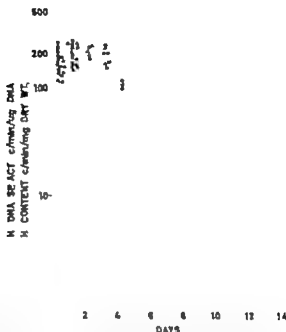


Fig. 1 H^3DNA specific activity and H^3 content of normal thymus in rats sacrificed at various intervals after single injection of H^3Tdr DNA extracted and counted in liquid scintillator as described previously (6). H^3 content determined by combustion of dehydrated thymus and collection of tritiated water

percentage of labeled small cells in the bone marrow could not have come there from the thymus. Furthermore, BIERRING found that marrow lymphocytes did not decrease in thymectomized rats (4) HULSE has pointed out that bone marrow lymphocytes recover much more quickly following whole body x irradiation than do peripheral lymphocytes (13) Although the cells may have migrated into the marrow from some other lymphoid area, the findings tend to support the view of OSMOND AND EVERETT that the marrow lymphocyte population is self-sustaining and has a rapid turnover If this small lymphocyte population in the bone marrow arises from proliferation within the marrow itself, it is clear that the peculiarly low mean grain count of these small lymphocytes, seen in the bone marrow and thymus, cannot be attributed to a lack of availability of the H^3Tdr to the progenitor cells because of the high labeling indices and mean grain counts of granulocytic and erythrocytic

progenitor cells. The low uptake per proliferating cell would have to be explained on some other basis, such as larger intracellular thymidine pools or reutilization of thymidine, thymine nucleotides, or DNA.

A relationship between the thymus and bone marrow has been suggested by several investigators through the years. One view ascribes a stem cell or trephocytic function to lymphocytes which increase in the marrow after such perturbations as whole body x-ray (13) accelerated erythropoiesis (20) and accelerated granulocytopoiesis (20) However the reverse relationship is suggested by the observations of KAPLAN et al. several years ago (14) They found that the mouse thymus recovered from x-ray depletion much more quickly if a portion of marrow was shielded or if normal marrow was given intravenously FORD AND MICKLEM in similar but more refined experiments provided unequivocal evidence that the cells which repopulate the thymus in such experiments are in bone marrow and appear to be lymphocytes (10) Such repopulation of the thymus did not derive from injected lymph node or thymus cells. These workers suggest the possibility that the thymus is continuously being reseeded from the bone marrow

The present findings support the view that bone marrow and thymus lymphoid cells have features in common. The presence of thymus-like lymphoid cells in the bone marrow and probably other parts of the lymphatic system, has relevance to the possible dissemination of thymic tissue and function If the population of thymus-like lymphocytes in the bone marrow once developed is self-sustaining, as the present data and studies of others suggest, then ablation of the thymus would be expected to have a variable and limited effect on the lymphatic system. Numerous studies in animals thymectomized after the neonatal period (11 17) support this view first elaborated by BEARD in 1903 (3)

Summary

Studies of the small lymphocyte population in the bone marrow of normal rats reveal large population of cells which shows the same kinetics of DNA labeling with tritiated thymidine and cell turnover as small lymphocytes in the thymus cortex. In contrast to peripheral lymphocytes, these thymus and bone marrow lymphocytes have very rapid rate of renewal and are derived from progenitors which have low uptake of tritiated thymidine into DNA. The findings support the view that the bone marrow contains self-sustaining, proliferating lymphoid cells which resemble those in the normal thymus cortex.

Résumé

L'étude des petits lymphocytes de la moëlle osseuse normale de rat démontre une forte population de cellules qui présentent au marquage à l'aide de thymidine tritiée le même métabolisme de l'ADN et le même turnover cellulaire que les petits lymphocytes du cortex du thymus. A l'encontre des lymphocytes périphériques les lymphocytes du thymus et de la moëlle se renouvellent très rapidement et dérivent de précurseurs qui n'incorporent que très peu de thymidine tritiée dans l'ADN. Ces résultats étayent l'opinion que la moëlle osseuse contient des cellules autonomes se reproduisant, qui ressemblent à celles de l'écorce normale du thymus.

Zusammenfassung

Untersuchungen der kleinen Lymphocyten im normalen Rattenknochenmark ergeben eine grosse Zellpopulation, die denselben DNA-Stoffwechsel bei Markierung mit Tritium-Thymidin und den gleichen Zellumsatz aufweisen, wie die kleinen Lymphocyten der Thymusrinde. Im Gegensatz zu peripheren Lymphocyten erneuern sich diese Lymphocyten von Thymus und Knochenmark sehr rasch und leiten sich von Vorstufen ab, die nur wenig Tritium-markiertes Thymidin in DNA einbauen. Die Ergebnisse bestätigen die Ansicht, dass das Knochenmark selbständige, proliferierende lymphoide Zellen enthält, die denjenigen der normalen Thymusrinde gleichen.

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Metabolic Responsiveness of Human Leukemic Leukocytes to Phagocytic Stimulation

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Since the work of Grafe (1) that white blood cells have a high oxidative metabolism, a number of studies have been published on the respiration and glycolysis of leukemic leukocytes (2–5). Especially Warburg's studies (6) on the metabolism of tumor cells have stimulated great interest in similar studies on the blood corpuscles. On the phagocytic potentialities of leukemic leukocytes, many investigations have been reported chiefly from the morphological point of view but the biochemical approach to this problem has not been systematically made.

Based on the reports from other investigators (7, 8, 9) and on our previous experiments (10, 11, 12), it is believed that during phagocytosis general metabolic activity of leukocytes is not increased but specific activation pattern is observed. Among the altered metabolisms increase of oxygen uptake and activation of hexose monophosphate shunt are remarkable, and these metabolic changes, within certain limits, are proportional both to the number of particles engulfed by the leukocytes and to the number of leukocytes used. The phase contrast microscopy showed that these changes occurred simultaneously with the phagocytic process (12). Accordingly, the process of this phagocytic stimulation may be employed for an assay method for phagocytosis. The biochemical studies on the human leukocytes in leukemias with special reference to their phagocytic activities will be presented in this paper.

Materials and Methods

Leukemic patients of various types who had peripheral leukocyte counts of more than 30,000 and accelerated red cell sedimentation rates were selected in order to

obtain leukocytes in highly purity and within short periods of time. Particularly with acute leukemia, there were selected only patients whose immature cells in peripheral blood made up more than 80% of leukocytes so that high homogeneity of blast cells was achieved in experiments with those cases. Dextran which serves for the acceleration of red cell sedimentation rate was added only in few instances. In most cases the blood was drawn before commencement of antileukemic chemotherapy. As anticoagulant, heparin was used in all cases. The number of cases which satisfied these conditions was accordingly limited.

For the classification and identification of the leukocytic types, the cytochemical staining methods such as Nadi oxidase, peroxidase, acid and alkaline phosphatase, PAS, and Sudan black B staining reactions were utilized as well as Giemsa stain, phase contrast microscopy electron microscopy and supravital stain. Grounds for the classification were as follows (13): in most cases of acute myeloic leukemias peroxidase positive immature cells were more or less encountered. When all the blast cells showed no peroxidase reaction, other cytochemical stainings and supravital stain along with phase contrast microscopy were made to differentiate between lymphoblast and myeloblast. Lymphoblasts showed no stainings specific to hematological granules such as peroxidase Sudan black B stain, Dopa oxidase and Dubinon staining and they had coarse nuclear membrane, indented nucleus and relatively large mitochondria as revealed by phase contrast microscopy. Cells of the monocytic series had the thin nuclear membrane with relatively large number of mitochondria scattered diffusely in the cytoplasm and with the prominent Golgi area, and some of them showed phagocytic activity. The large neutral red rosette formation was found in monocytic series of cells.

The separation of leukocytes from whole blood was accomplished by the following procedures: the leukocyte-rich plasma was removed from whole blood after standing for 30–60 min at room temperature. Of few cases with lower red cell sedimentation rate, the plasma was separated by low speed centrifugation. The separated plasma was then centrifugated at 1,000 rpm for 4 min. The supernate which contained large number of platelets was removed and the sedimented leukocytes were washed and suspended in the Krebs-Ringer phosphate buffer (pH 7.4) at an appropriate concentration (7–10 × 10[?] /ml). In phagocytosis experiments autologous serum was added at final concentration of 10%. Cell counting was performed with the hemocytometer in duplicate and differential counting of leukocytes was made on stained smear. Dry weight was determined in most cases colorimetrically according to the method of BAILEY AND N JAD (14): 2 ml of dichromate reagent (2% potassium dichromate in sulfuric acid) was added to 1 ml of the cell suspension (containing 0.2–2.0 mg dry weight) and the color developing after 30 min incubation in boiling water was read against reagent blank at 580 mμ.

Phagocytosis: Heat-killed (80 °C, 30 min) staphylococcus aureus (coagulase positive Terajima strain), staphylococcus albus (coagulase negative), or vinyl chloride particles, 1–2 μ in diameter were used for phagocytic experiments. Since no difference in the phagocytic process was found between two strains of staphylococcus in the presence of autologous serum, staphylococcus aureus was used in most experiment.

Respiratory experiments: Oxygen uptake was measured in the WARBURG apparatus.

Glycolysis: Measurements of aerobic glycolysis were carried out in plastic test tubes set on metabolic shaker. Anaerobic glycolysis was carried on in the WARBURG's apparatus, gas phase of which consisted of 95% nitrogen and 5% carbon dioxide.

Experiments with labeled glucose. Carbon[14] dioxide produced by the oxidation of C^{14}-labeled glucose was absorbed in potassium hydroxide in the center well of the WARBURG vessel, trapped as bicarbonate and then liberated by addition of 30% trichloro-acetic acid which also served to terminate the reaction. The carbon dioxide was precipitated as barium carbonate in the presence of the carrier, plated on stainless

planchet, and the radioactivity assayed using thin window gas-flow counter The results were corrected for background and for the infinite thinness of the barium carbonate layer

Lipid metabolism. The rate of incorporation of sodium acetate-1-C^{14} into the leukocytic lipid fractions was determined in 20 ml Erlenmeyer flask. Leukocyte suspension (about 3×10^8 cells in 5 ml Krebs-Ringer phosphate buffer) was incubated with 1 μc of sodium acetate-1-C^{14} (specific activity 6.7 mc/m mole) 20 μ moles glucose, 30 μ moles of carrier sodium acetate and 1 ml autologous serum in the presence or absence of bacilli.

The extraction of lipid was accomplished according to the method of FOLCH et al. (15) Counting of radioactivity in the lipid fraction was carried out in Packard Tri-Carb liquid scintillation spectrometer model 314 E. Separation of phospholipids from neutral lipids was performed by the method of EDER (16)

Lysozyme was assayed by the optical density decrement of micrococcus lysodeicticus.

Acid and alkaline phosphatases were determined by the method of BESSEY et al. (17) using disodium-p-nitro-phenyl-phosphate as substrate.

Chemical determinations. Glucose was assayed by the method of YAMAMOTO (using anthrone as reagents) (18) Lactic acid was determined by the method of BARKER AND SUMMERSON (19).

Results

Cell number and dry weight The metabolic rate expressed per cell unit has some limitations because of the difference in volume among cells from individual leukemic cases. The rate evaluated in terms of per dry weight cell phosphorus or cell nitrogen may better represent the specific activities of cell metabolism. However different degrees of contamination with other types of blood cell due to more or less incomplete separation would make the results somewhat equivocal. In this study the rate will be both expressed

Table I
Relations between cell number and dry weight.

Type of cells	Number of cases	Number of cells per mg dry weight** (x 10⁶)	Tests of significance
AML	8	7 40 ± 1.67*	
CML	6	7 49 ± 1 72	
CLL	4	14 0 ± 1.55	AML versus CML no difference
ALL	2	10.4 ± 1 13	AML and CML vs CLL: $p < 0.001$
ML	2	10.0 ± 1 [illegible]	AML vs ALL: $p < 0.05$
Normal	3	9 41 ± 0.42	CLL Normal $p < 0.05$
Red Cells	3	27.5 ± 1.50	

AML acute myeloic leukemia. ALL: acute lymphatic leukemia. ML: monocytic leukemia. CML chronic myelocytic leukemia. CLL chronic lymphatic leukemia. Standard deviation of the mean.

1 mg dry weight was equivalent to OD_{660} 0.230 ± 0.011

Table II

Oxygen consumption of normal and leukemic leukocytes and changes in its rate during phagocytosis.

Type of leukemia	Number of cases	Oxygen uptake in μmole/10^{10} hr	(μ mole/mg*hr)	Per cent increase of oxygen uptake in the presence of bacilli
AML	4	0.386 ± 0.078	(0.245)	+ 21.3 ± 7.7
ALL	3	0.198 ± 0.009	(0.200)	+ 25.9 ± 3.1
ML	4	0.[illegible]17 ± 0.085	(0.[illegible]14)	+ 66.0 ± 22.2
CML	11	0.180 ± 0.004	(0.146)	+ 91.9 ± 76.4
CLL	2	0.139 ± 0.002	(0.178)	+ 54.3 ± 2.4
Normal	3	0.495 ± 0.049	(0.465)	+216.4 ± 53.0

See table I.
** Standard error of the mean

	Oxygen uptake		Effect of phagocytosis on the increase of oxygen uptake	
Test of significance	AML vs ALL	$p < 0.1$	AML vs CML	$p < 0.05$
	AML vs CML	$p < 0.1$	CML vs normal	$p < 0.03$
	AML vs ML	$p < 0.2$	AML vs normal	$p < 0.01$
	AML vs normal	$p < 0.3$	AML vs ML	$p < 0.15$
	CML vs normal	$p < 0.001$	CML vs CLL	$p < 0.2$
	CML vs CLL	$p <$ 0.[illegible]	normal vs ML	$p \leq 0.01$
	normal vs CLL	$p < 0.001$	normal vs CLL	$p < 0.01$
	ALL vs CLL	$p < 0.1$	ALL vs CLL	$p < 0.01$
	normal vs ML	$p < 0.05$		

per 10^{10} cells and referred to dry weight. In most preparations the ratio of erythrocyte to leukocyte was less than one. The colorimetric dry weight determination was found to be simple and useful in such a cell suspension and the applications of this method to leukemic leukocytes are presented in table I. No significant difference in dry weight between acute myeloic and chronic myelocytic leukemias was found and the cellular volumes of both types were nearly double those of chronic lymphatic leukemia (20)

Respiratory rate of leukocytes from each type of leukemias and its changes caused by phagocytosis As revealed from table II, oxygen consumption per 10^{10} cells was highest in normal leukocytes and the others ranked in the following order acute myeloic, monocytic, acute lymphatic or chronic myelocytic and chronic lymphatic leukemia. The large standard deviations of the mean value of oxygen uptake in the acute myeloic and monocytic leukemias may reflect the aberrant metabolism of immature cells varying with cases. In contrast, the relatively small deviations of the results in

chronic myelocytic and chronic lymphatic leukemias may be due to metabolic uniformity as well as constant preservation of cellular activity after the various manipulations in the separating procedures.

The increment in oxygen uptake in the presence of bacilli was most marked, as was expected in normal leukocytes. The leukocytes in chronic myelocytic leukemia showed a moderate enhancement and acute myeloic and acute lymphatic leukemia only a slight increase in oxygen uptake which was attributed to the small number of mature leukocytes owning phagocytic activity. In fact, the stained smear revealed that the band and segmented neutrophils, showing more or less abnormal appearance, ingested large number of bacilli and caused increased oxygen uptake 3 to 7 folds as calculated from the percentage of phagocytizing neutrophils in total leukocytes and from the increment in oxygen uptake. These situations were confirmed from the observation on one case in which myeloblasts made up more than 99% of total leukocytes, and no increment in oxygen consumption occurred by addition of bacilli to the leukocyte suspension. In the case of monocytic leukemia a moderate increase, which is greater than acute myeloic but less than chronic myelocytic leukemia cases, was observed. That the increased oxygen uptake was partly caused by the phagocytic activity of monocytic cells was assumed based on the observations of stained smear. In a case of idiopathic eosinophilia (a male 21 years of age, hemoglobin 12.8 g/%, leukocyte counts up to 45 800 per mm³ with 89% of eosinophils) pronounced increase up to 7 folds was noted and the stained smear revealed that almost all eosinophils had engulfed large numbers of bacilli.

Table III
Lactate production of normal and leukemic leukocytes.

Type of leukemia	Number of cases	μ mole/10⁸/hr	(μ mole/mg)	Test of significance	
AML	4	0.339 ± 0.120*	(0.320)	AML CML	$p < 0.2$
CML	3	0.646 ± 0.141	(0.370)	AML vs normal	$p < 0.1$
ALL	2	0.232 ± 0.074	(0.235)	CML vs CLL	$p < 0.06$
CLL	2	0.129 ± 0.013	(0.174)	normal vs CLL	$p < 0.01$
Normal	4	0.725 ± 0.128	(0.712)	ALL CLL	$p < 0.4$

See table I
Standard deviation of the mean.

Table IV

Radioactive CO_2 liberation from glucose-1-C^{14} and glucose-6-C^{14}

Type of leukemia	Number of cases	Glucose-1-C^{14} → $C^{14}O_2$ (μ moles/10^{10}/hr)		Glucose-6-C^{14} → $C^{14}O_2$ (μ moles/10^{10}/hr)		C_1/C_6 ratio	
		Particle		Particle		Particle	
		(−)	(+)	(−)	(+)	(−)	(+)
AML	7	14.85±4 23	51.3±26.8	4.60±1.50	7.69±5.10	4 4±1.00	7.2±1.2
		p <0.005		p <0.15		p <0.05	
CML	4	20.94±7 47	167 4±75.4	2.37±0.69	6.37±1.68	8.8±1.20	22.9±6.0
		p <0.1		p <0.05		p <0.05	
CLL	1	6.14	42.0	1.02	2.84	6.0	14.8
Normal	4	23.15±6.45	216 0±65.5	2.43±0 84	7.60±1.81	9.5±1 40	28.5±5.8
		p <0.03		p <0 05		p <0.05	
Tests of significance							
AML vs CML		p <0.4	p <0.3	p <0.3	—	p <0.05	p <0.1
AML vs norm.		p <0.4	p <0.1	p <0.3	—	p <0.05	p <0.05
CML vs norm.		—	—	—	—	—	p <0.5

See table I.
Standard error of the mean.
p values indicate differences in the presence or absence of added bacilli.
p refers to the probability value for differences between two systems.

Glycolysis In table III there is shown the aerobic glycolysis of leukocytes in leukemias. Normal mature leukocytes exhibited the highest activity and it is thought that the more mature the cells, the lactate production tends to be the greater Lymphatic cells exhibited lower activity of aerobic glycolysis and the small lymphocytes predominating in chronic lymphatic leukemia showed little glycolytic activity under aerobic condition.

In view of the increased glucose oxidation via hexose monophosphate shunt during phagocytosis as shown in neutrophils of other sources (7 12) it was of interest to make a study on this problem in leukemic leukocytes. Such relations, as presented in table IV were found to be present for the leukemic leukocytes. The liberation of radioactive CO_2 from glucose labaled in carbon 1 was markedly enhanced in normal and chronic myelocytic leukemia cases in proportion to the number of leukocytes which had ingested bacilli. The ratio of counts appearing in expired CO_2 (C-1/C-6 ratio) increased from a value of 9.5 at rest to 28.5 in normal and from 8.8

highest in normal mature leukocytes and decreased in activities in proportion to the immaturity of the cells.

On the contrary lipid turnover rates were inversely proportional to cell maturity In view of the greater susceptibility of mature leukocytes to injury and greater adhesiveness of them to the surface of glass, these data may suggest that lipid metabolism is highly sensitive to injury in contrast to the aerobic glycolytic activities which are even augmented in the same condition. In this connection, the work of MARKS et al (26) is interesting in that the separation of leukocytes before incubation was associated with an 80% decrease in the rate of lipid synthesis compared to that of leukocytes separated from whole blood subsequent to incubation. However based on the data shown in table V together with the relatively high oxidative metabolism of the blast cells as presented in table II it would appear that lipid metabolism actively proceeds in the immature cells and provides a part of energy required for cellular proliferation and differentiation

Lymphatic cells showed lower activities in both the aerobic glycolysis and the lipid metabolism than those of the myeloic cells even when the smaller volume of the lymphocytes than the myeloic cells was taken into consideration. The results mentioned above are consistent with the results by KIDSON (27–29) using whole blood leukocytes where myeloic cells exhibited higher lipid metabolism than the lymphatic ones the acute myeloic leukemia having shown the greatest activity Increased oxygen uptake and activation of hexose monophosphate shunt were observed on addition of bacilli in chronic myelocytic, monocytic, chronic lymphatic and acute myeloic leukemias in the decreasing order The phagocytic stimulation was proportional to the number of leukocytes capable of phagocytosis. Myeloic cells passing the maturing stage of metamyelocyte exhibited phagocytic activities and resulted in the 3 to 7 folds increase of oxygen consumption. These results suggest that in the leukemia cases the leukocytes are endowed with phagocytic activity and with enzymatic arrangements capable of responding to the phagocytic stimulation and the activity is decided by maturity of the cells.

In view of the different responsiveness to addition of foreign particles and varied metabolic patterns in each type of leukemias, it will be of interest to apply these metabolic properties to the

classification and identification of leukemic cases. Though additional data are to be accumulated, the biochemical studies will provide some important clues to the clarification of the characteristics of leukemic cell metabolism.

Summary

Metabolic activities of human leukocytes obtained from normal blood and various types of leukemia and the metabolic changes by the addition of bacilli were studied. Increase of oxygen uptake as well as the activation of hexose monophosphate shunt were observed during phagocytosis of normal leukocytes. These metabolic changes was highest in normal leukocytes and varied in degree with the different types of leukemic leukocytes, depending upon the number of cells capable of phagocytosis. The more mature the cells, glycolytic rates were the greater. On the contrary the lipid turnover rates were inversely proportional to cell maturity. Lymphatic cells exhibited low metabolic rates of glycolysis and lipid turnover.

Résumé

Chez des personnes saines et chez différents types de leucémiques le métabolisme des leucocytes humains, ainsi que ses changements sous l'effet de bactéries, ont été étudiés. Les leucocytes normaux présentent pendant la phagocytose une consommation augmentée d'oxygène et une activation du shunt de monophosphate d'hexose. Ces changements métaboliques sont le plus prononcés chez les leucocytes normaux et varient de degré chez les différents types de leucocytes leucémiques, selon le nombre de cellules susceptibles de phagocytose. Plus les cellules sont mûres, plus la glycolyse est forte. Par contre, la vitesse du turnover des lipides est inversement proportionelle à la maturité des cellules. Les cellules lymphatiques ont une vitesse de glycolyse et un métabolisme des lipides moindres.

Zusammenfassung

Der Stoffwechsel menschlicher Leukozyten von Gesunden und von verschiedenen Leukämietypen und seine Beeinflussung durch Zusatz von Bakterien wurde untersucht. Normale Leukozyten zeigten während der Phagozytose einen gesteigerten Sauerstoffverbrauch und eine Aktivierung des Hexosemonophosphat-Shunt. Diese Stoffwechselveränderungen waren am stärksten bei normalen Leukozyten, bei leukämischen Zellen ergaben sich Schwankungen in Abhängigkeit von der Zahl phagozytosefähiger Zellen. *Je reifer die Zellen waren, desto stärker war ihre Glykolyse. Im Gegensatz* dazu war der Lipidumsatz umgekehrt proportional zur Zellreife. Lymphatische Zellen zeigten niedrigere Werte von Glykolyse und Lipidstoffwechsel.

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Mechanism of Prednisolone-Induced Leucocytosis in Man

By I. Straub, E. Kékes and Ágnes Szebeni

Treatment with ACTH, cortisone or metasteroids may give rise to leucocytosis which does not fit into the clinical picture of the primary disease and may therefore lead to diagnostic difficulties. Its mechanism is still somewhat obscure: depletion of white-cell depots, the appearance of a humoral factor in the blood and increased leucocytopoiesis (11), retention of white cells in the vascular bed (1) are the best known factors that may explain this type of leucocytosis.

The role of a humoral factor was suggested by earlier investigations of the present authors, in which serum, obtained at various intervals from 12 patients treated with prednisolone, was injected into mice. Sera collected from 4 patients on the 4th or 5th day of prednisolone treatment provoked significant leucocytosis in the test animals, whereas serum collected in an earlier or later phase of the treatment did not influence the white count more than normal serum. From a patient, treated for agranulocytosis in 1961, blood was drawn before, then on the 7th and on the 18th day of the prednisolone treatment. Only the serum collected on the 7th day induced significant leucocytosis in the test mice (13). These observations led to the conclusion that the humoral factor responsible for leucocytosis appears in the blood of the patients in a certain phase of prednisolone treatment only. Therefore, in all subsequent cases we collected serum from the patients and inoculated different groups of mice with it on every day from the beginning of the prednisolone treatment.

Material and Methods

The sera of [illegible] patients were used for inoculation. Six were being treated for bronchial asthma, 5 for rheumatic fever, 3 for agranulocytosis and one, each, for subacute

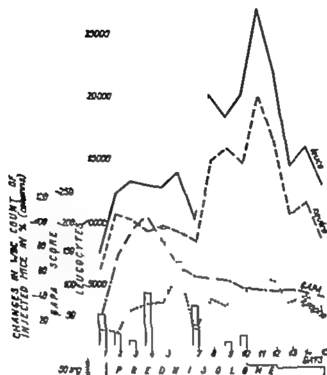


Fig 1 A. Z., 26 years old, female with rheumatic fever Changes in the patient cir culating leucocytes along with the changes of GAPA values during prednisolone treat ment, further percentual increases—during the same time—in the leucocyte count of mice inoculated with the patient serum.

bacterial endocarditis, hirsutism, multiple sclerosis and spondylosis. The patients received daily dose of 50 mg prednisolone during 7 to 10 days, thereafter the dose was gradually reduced.

During the first 10 to 14 days, the patients were daily examined for white-cell count, qualitative blood picture and the granulocytic alkaline phosphatase activity (GAPA) the latter by the method of KAPLOW (8)

The leucocytotic effect of the patients sera on the mice was determined by the method of DOCTOR AND KELEMEN (4) W collected blood every day and injected intravenously 0.2 ml of the serum into 7 animals each. The number and qualitative composition of the white cell were determined before the inoculation and t 2,3 and 5 days thereafter The normal white cell count of the animals varied from 10,000 to 20,000. W accepted the presence of leucocytotic humoral agent in the serum only if the inoculation induced more than 50% rise in the white count, if it occurred at least in 5 of the 7 test animals, and if it induced characteristic change in the qualitative blood picture (the latter to be detailed further below) The rise in leucocyte count provoked by serum collected prior to prednisolone treatment amounted, on an average to 20%, and was accepted as the control value

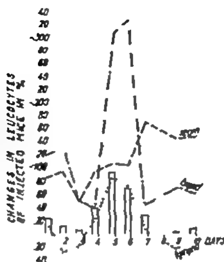


Fig 2 Quantitative (columns) and qualitative changes in the leucocytes of mice inoculated with the serum collected from patient in fig 1

Results

Results are illustrated in figs. 1–4

It can be seen from fig 1 that the white cell count of A.Z. (female patient, 26 years of age) treated for rheumatic fever rose considerably after the first day of treatment with prednisolone. The rise was due to an elevation of the number of neutrophils. Although the total white count still rose slightly during the next 4 days, the number of neutrophils gradually decreased in this phase, so that it was also the gradual increase in the number of lymphocytes which took place in the maintenance of leucocytosis from the 3rd day The lymphocyte number amounted to 5 times the original value on the 6th day Both the neutrophil and the lymphocyte count and so also the total white count diminished on the 7th day The white count increased twofold from the 7th to the 8th day phenomen due to corresponding increase in the number of both neutrophils and lymphocytes. A peak of the white count was reached on the 10th day when the number of neutrophils amounted to more than 9 times, that of the lymphocytes to 5 times, the initial value. The dose of prednisolone was gradually reduced thereafter There followed a rapid parallel decrease in the number of neutrophils and lymphocytes, with cor responding fall of the total leucocyte count. The 'score' indicating GAPA showed considerable increase after the first day reached maximum on the 3rd day of prednisolone treatment and underwent gradual decrease until the 10th day when it became stabilized at a level somewhat bove the original value. There was an increase in the number of metamyelocytes on the 6th day and myelocytes appeared in the peripheral blood as from the 7th day

As is evident from fig 2, it was the serum collected on the 4th and the 5th day of prednisolone treatment which provoked more than 50% increase in the white cell count of the test animals. The increase was chiefly due to band forms, as also to segmented neutrophils and lymphocytes. Sera collected on the other days either before or after provoked but moderate leucocytosis the neutrophil count increased slightly without a

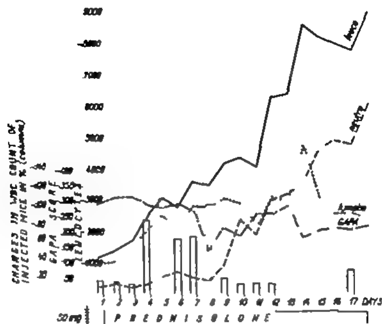


Fig. 3. J. B., 52 years old, male, suffering from plasmocytoma complicated by agranulocytosis during course with Melphalan. Changes in the patient's circulating leucocytes along with the changes of GAPA values during prednisolone treatment, further percentual increases—during the same time—in the leucocyte count of mice inoculated with the patient's serum.

change in the ratio between band and segmented neutrophils the number of lymphocytes showed either moderate increase or decrease.

Fig. 3 illustrates the data of J. B., male patient of 52 years. Suffering from multiple myeloma, he was treated with Melphalan agranulocytosis supervened, and the patient was given prednisolone. After 3 days of prednisolone treatment the white cell count gradually reached the double of its initial value owing to the increase in the number of lymphocytes. The rise of the total leucocyte count went on in the next days; besides continued rise of the lymphocyte count even slight increase in the number of neutrophil granulocytes was observed. The rise in the number of neutrophils became parallel to that of the total white cell count as from the 9th day A transitory reduction in the number of lymphocytes was followed by new maximum on the 14th day after which gradual diminution was observed. Compared to the high initial value, the 'score' of GAPA showed moderate diminution after the first week, but remained still fairly high during the whole time of observation. There was an increase in the number of metamyelocytes after the 9th day of prednisolone treatment, and myelocytes and normoblasts appeared in the peripheral blood.

Fig. 4 makes it clear that it was only the blood sample obtained from the agranulocytotic patient between the 4th and 8th day of prednisolone treatment, which provoked significant leucocytosis in the inoculated mice. It was due to an increase in the number of neutrophil granulocytes and lymphocytes. Also the number of young neutrophils increased very considerably on the 5th day Sera collected on other days induced but

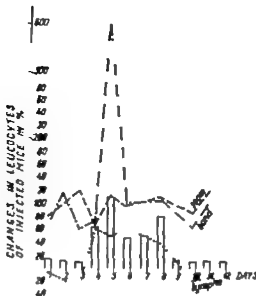


Fig 4 Quantitative (columns) and qualitative changes in the leucocytes of mice inoculated with the serum collected from patient in fig 3.

moderate rise in white count. It was due to small increase in the number of neutrophils with a simultaneous reduction of lymphocytes.

General survey of the observations made in the 18 cases under review

1 Leucocytosis was induced in humans by the administration of prednisolone there was a prompt increase in the number of neutrophils and usually one after the 3rd day in that of lymphocytes.

2. The leucocytosis reached the first peak after 2 to 4 days. The white cell count decreased thereafter more or less considerably and then rose gradually once more to reach a second peak between the 5th and 10th day of the treatment. This was followed by a second decrease, but the number of white cells did not descend to the original level during the treatment.

3 Changes in the number of neutrophils were in proportion to those in the total count. The number of lymphocytes usually decreased during the first 2 days, invariably increased thereafter and reached in 3 to 4 days the double to five fold of their original value, usually at a time when the leucocytosis had already begun to diminish. The lymphocyte count diminished during the next 2 to 4 days but still remained above the initial value except in one case,

it rose once more together with the second increase in the degree of leucocytosis and reached in some cases 500 per cent of the original level. Along with the decrease of the leucocyte count also the number of lymphocytes diminished.

4 No shift to the left was observed in the qualitative blood picture at the time of the first peak of the leucocytosis, while a pronounced shift to the left appeared after the second peak there appeared metamyelocytes usually also normoblasts.

5 The GAPA score showed a considerable increase at the first peak of leucocytosis. It rapidly decreased thereafter in most cases, and reached the initial value on the 5th or 6th day In other cases it became stabilized above the initial level.

6. It was, as rule, with sera collected on the 3rd to 5th day prednisolone treatment that significant leucocytosis could be provoked in the test animals. Increases in white count produced by sera collected earlier or later were not more pronounced than those following inoculation with normal serum.

7 Augmentation of the circulating leucocytes in mice exceeding 50 / was as a rule chiefly the result of an increase in band neutrophils, but also the increase in the number of segmented neutrophils and lymphocytes contributed to it. Normal reaction involves no essential change in the ratio of young cells ans consists in neutrophil granulocytosis which is usually accompanied by a diminution of the lymphocyte count.

8 In the investigated cases of agranulocytosis there was no initial increase in the neutrophil count, nor intensified GAPA, while other manifestations were as described in the foregoing

Discussion

It is commonly known that ACTH, cortisone and metasteroids, among others, produce leucocytosis and neutrophilia (7 9 10 17) This effect is thought by Shen and Hoshino (11) to be initiated by a mobilization of leucocyte depots. They found that, after the supposed depletion of the depots of the experimental rats, leucocytopoiesis was increased, a phenomen demonstrated by them to have been caused by the action of a humoral agent.

The curve of the white cell count of our human patients showed 2 waves with 2 peaks in the present investigations. The first maximum must have been due to leucocytes released from depots. This

assumption is supported by the following observations 1 The white cell count increases immediately a phenomen which—in consideration of the shortage of time—refers to the contribution of an already available white cell pool 2 The increase in the number of neutrophils occurred without a shift to the left. 3 Rapid and significant increase in the GAPA score a phenomenon showing that aged cells passed into the blood stream, since intensified GAPA appears to be a sign of physiological maturity (16) TRUBOWITZ et al. (14 15) state that an intensification of the GAPA in cases of leucocytosis suggests the circulation of cells recently released from storage sites. 4 While there was always a first wave in the white cell count after the administration of prednisolone when haemopoiesis was normal, *it did not appear in agranulocytosis since there is no depot* in such cases.

That the second maximum was due to increased leucocytopoiesis seems to be substantiated by the following phenomena 1 It appeared after a few days of prednisolone treatment, this period being necessary for the formation and release of new granulocytes (3) 2. A shift to the left could be observed in the qualitative blood picture the appearance of metamyelocytes and myelocytes was indicative of intensified and accelerated leucocytopoiesis. 3 At the time of the second wave the GAPA was approximately the same as the original or became stabilized at a slightly higher level, a phenomen apparently pointing to the increased formation of fresh cells. Rise in the white cell count associated with diminished GAPA points to the outflow from the bone marrow of 'young' cells *with lower enzyme content (14 15) 4 The second wave of leucocytosis* which appeared after the administration of prednisolone, appeared also in the cases of agranulocytosis owing to the restoration and then to the increased rate of cell production.

It has been demonstrated that in leucocytosis the blood contains a humoral factor responsible for the increase in the number of white cells (2 5 6 12) We regard the significant leucocytosis, induced in mice by the serum of patients treated with prednisolone, as a manifestation of the action of the humoral factor under consideration. In cases of leucocytosis provoked in mice by normal human serum the increase in white cell count remains below 50% and consists in an increase of the number of neutrophils without any essential change in the ratio between young and mature cells, while the lymphocyte count usually decreases. If leucocytosis is due to

inoculation with serum containing the 'humoral factor' in question, the increase in white count exceeds 50 and mostly reaches 100% there is a shift to the left in the differential count and the number of both neutrophils and lymphocytes increases. The effect of the 'humoral factor' thus reveals itself not only through a higher degree of the leucocytosis but through a characteristic change in the differential blood picture as well. The agent under review arises and disappears between the two peaks of leucocytosis, i.e. its presence can be demonstrated only at a definite point of time and only for a few days. The time table of its appearance is similar in agranulocytosis. That the agent is probably involved in the starting and perhaps also in the maintenance of the increased formation of white blood cells seems to be proved by the time between its demonstrable appearance and the second peak of the leucocyte curve, an interval of about 5 days. It has been shown that this is the time necessary for the peripheral manifestation of an acute stimulus which caused granulocytopoietic proliferation (5)

Summary

Experiments in human subjects showed that the leucocytosis, induced by prednisolone, consists primarily of white cell release from the depots and then (after the temporary appearance of "leucocytopoietic humoral factor") is maintained by an increase in white cell formation.

Résumé

Des expériences ont pu démontrer chez l'homme, que la leucocytose, provoquée par la prédnisolone, consiste d'abord en une libération de cellules des dépôts et qu'elle est ensuite maintenue (après l'apparition temporaire d'un facteur leucocytopoétique humoral) par une augmentation de la leucocytopoïèse.

Zusammenfassung

Beim Menschen wurde experimentell nachgewiesen, daß die durch Prednisolon hervorgerufene Leukozytose zunächst durch eine Zellabgabe aus den Depots bedingt ist und dann (nach vorübergehendem Auftreten eines humoralen «leukopoietischen Faktors») durch eine Steigerung der weissen Blutzellbildung aufrecht erhalten wird.

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X-Chromosomenanomalie und Ahaptoglobinämie bei Hämophilie A

Von R. Witkowski, H. Zabel und G. Bundschuh

Seit fast 30 Jahren kennt man als eine der Ursachen der Bluterkrankheit einen Mangel an antihämophilem Globulin (AHG). Die Bezeichnung Faktor VIII ist dafür international gebräuchlich, womit die verwirrende Nomenklatur von 12 auf 2 Synonyma eingeengt wird. Für den vielphasischen Ablauf der Blutgerinnung werden von den verschiedenen Forschergruppen noch unterschiedliche Einteilungen gebraucht, obwohl das Krankheitsbild gerinnungsphysiologisch weitgehend erforscht ist (8, 19), hingegen sind Probleme genetischer Art offengeblieben.

Hinweis auf den X-chromosomalen Erbgang gibt schon die Erbfolge, nämlich das Auftreten der Blutungen als phänotypisches Merkmal beim männlichen Geschlecht. Demgegenüber stellt dieses Phänomen beim weiblichen Geschlecht eine Rarität dar, denn sowohl Vater als auch Mutter müssen belastet sein. Schwerste Formen stellen entsprechend dem Homozygotie-Effekt Letalfaktoren dar. Bei genetischen Studien des Hämophiliemerkmals besitzt die Konduktorin ein besonderes Interesse, die hinsichtlich der Geschlechtschromosomen homozygot XX, aber in bezug auf das hämophiliesteuernde Gen heterozygot X'X ist. Sie weist gerinnungsphysiologisch ebenfalls einen Faktor-VIII-Mangel auf, ist aber keine «Bluterin». Darüber hinaus beschäftigt uns das Problem eines verminderten Faktors V bei weiblichen Familienangehörigen von Blutern. Heinrich (4, 5) sah bei Koagulopathien wiederholt Kombination eines Faktor-VIII-Mangels mit Faktor-V-Verminderung. Der Autor zitiert in diesen Arbeiten die Interpretation von Lehmann (9), der Genmutation und Ausfall des normalen Gen

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Durch *Blutgruppenbestimmungen* unseres Probanden und seiner nächsten Angehörigen erfaßten wir eine latente Ahaptoglobinämie. Die Ahaptoglobinämie unseres Hämophilie-A-Patienten *zeigte sich nur in der akuten Blutungsphase* (Hb 4,5 g%), später war ein normaler Hp 2-1 Typ darstellbar (Abb. 1)



Abb. 1 Darstellung der Haptoglobinmuster des Patienten J. M. mit Hämophilie A. Oben Hp-Typ 2-1 (subakute Phase). Unten Ahaptoglobinämie (akute Blutungsphase) Auftrennung der Serumproben in der Stärkeelektrophorese Trispuffersystem, pH 7,6 Trennzeit 6 Stunden.

Haptoglobin ist ein Bestandteil der Alpha₂-Fraktion und wurde 1938 erstmalig von JAYLE u. a. beschrieben. Es besitzt die Fähigkeit, Hämoglobin zu binden. Der Komplex Hb–Hp wird durch die gesunde Niere infolge seiner Molekulgröße (max. 310 000) nicht ausgeschieden. Beim Fehlen von Haptoglobin im Serum, also bei Ahaptoglobinämie, folgt Hämoglobinurie. Der Anteil der Hämoglobinrückresorption ist offenbar minimal (19); zusammenfassende Literaturübersicht (15). Die weitgehende Abklärung der genetischen Determination des Hp-Systems erfolgte durch SMITHIES (18). PROKOP und BUNDSCHUH (15) unterscheiden zwischen einer genetisch bedingten primären Ahaptoglobinämie, einer sekundären, durch Krankheit bedingten und einer bei Säuglingen vorkommenden Form. Bei unserem Patienten handelt es sich um eine latente, sekundäre Ahaptoglobinämie, deren Ursache eine Hämoglobinresorption in den Hämatomen nach massiven Blutungen ist. Bei diesen temporären Ahaptoglobinämien überwiegt die Eliminierung des Hp–Hb-Komplexes die Neubildungsrate.

Wir führten bei unserem Bluter und seinen nächsten Angehörigen Chromosomenanalysen aus dem Blute durch. Nach der Methode von MOORHEAD et al. (12) wurden die Chromosomen aus Leukozyten dargestellt und nach dem DENVER-Schema (2) geordnet. Wir fanden bei allen untersuchten Personen 46 Chromosomen in den Metaphaseplatten. Auffällig war bei unserem Hämophilie-A-Patienten und den beiden Konduktorinnen ein Chromosom, das mit einer relativen Länge von 80,7 (Gesamtlänge des haploiden Autosomensatzes = 1000) der Gruppe A des DENVER-Schemas zuzuordnen war (Abb. 2, 3). Gegen eine Trisomie 1–3 sprach jedoch die Lage des Zentromers in dem abnormalen Chromosom. Während der Armindex, d. h. das Verhältnis der Länge des langen zum kurzen Arm in der Gruppe A normalerweise 1,1–1,6 beträgt, liegt bei diesem Chromosom ein Index von 3,0 vor. Da weiterhin der Gruppe C (6—12+X) jeweils ein Chromosom fehlte, ist das fragliche Chromosom dieser Gruppe, und zwar als abnormales X-Chromosom, zuzuordnen.

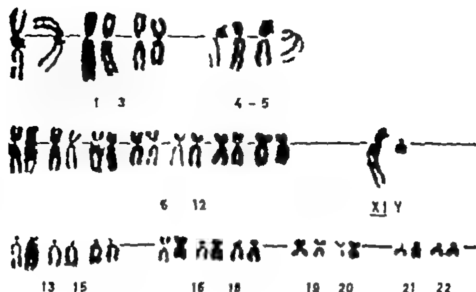


Abb. 2 Idiogramm des Hämophilie-A-Patienten J. M. X-Chromosom abnormal verlängert.

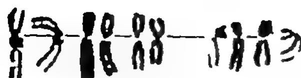


Abb. 3 Idiogramm einer Konduktorin, Mutter des Hämophilie-Patienten J. M.
Ein X-Chromosom deutlich verlängert

Diskussion

Der rezessive X-chromosomal gebundene Erbgang der Hämophilie A ist seit langem bekannt und gilt als eines ihrer zuverlässigsten Merkmale. Wenn man also im Idiogramm nach einer morphologischen Manifestation der Krankheit fahndet, wird man vor allem das X Chromosom untersuchen müssen 1962 berichten KOSENOW UND PFEIFFER (7) bei der Hämophilie seien keinerlei Chromosomenabnormitäten gefunden worden. Im gleichen Jahr beschrieben ELVES UND ISRAELS (3) ein abnormal langes Chromosom bei einem Fall von Hämophilie, das von den Verfassern als X-Chromosom angesehen wurde Allerdings wies der betreffende Patient außer dem Blutungsübel noch weitere kongenitale Mißbildungen auf. Es lag also nahe, die gefundene Chromosomenaberration in erster Linie für die Malformationen verantwortlich zu machen und die Hämophilie nur in indirektem Zusammenhang damit oder überhaupt isoliert davon zu sehen. Wir hielten es daher für angebracht, die Chromosomen der von uns beobachteten Familie mit Hämophilie, deren Mitglieder keinerlei andere Mißbildungen erkennen lassen, zu untersuchen. Da Form und Länge des von ELVES UND ISRAELS gefundenen Chromosoms genau mit den unseren übereinstimmen, schließen wir bei dem X Chromosom mit verlängertem Arm auf einen ursächlichen Zusammenhang mit der Hämophilie Zur Klärung der Entstehung dieser Anomalie werden noch weitere Merkmalsträger untersucht werden müssen. Wahrscheinlich liegt eine während der Oogenese bzw. Spermiogenese entstandene Translokation oder Reduplikation eines Chromosomenabschnittes vor Die Schleimhautabstriche der Familienangehörigen unseres Bluters entsprachen dem Geschlecht, d. h. nur bei den Konduktorinnen waren Barrkörper vorhanden.

Von genetischem Interesse ist weiterhin die Frage, warum trotz des rezessiven Erbgangs bei den Konduktorinnen, die im heterozygoten Zustand theoretisch hinsichtlich der Hämophilie vollkommen merkmalsfrei sein müßten, Blutgerinnungsanomalien im Sinne einer unterschwelligen Hämophilie feststellbar sind. Es wird in diesem Zusammenhang von einem semidominanten Erbgang oder von einer unvollständigen Penetranz des Hämophiliegens gesprochen (16) Eine Erklärung mag in der besonderen Stellung des X Chromosoms im weiblichen Chromosomensatz zu finden



1 – 3 4 5



6 – 12 XI Y



13 15 16 18 19 20 21 22

Abb. Idiogramm des Hämophilie-A-Patienten J. M. X-Chromosom abnormal verläng



1 – 3 4 5





6 12 XI



13 15 16 – 18 19 20 21 22

Abb. 3. Idiogramm einer Konduktorin, Mutter des Hämophilie-Patienten J. M. Ein X-Chromosom deutlich verlängert

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Der rezessive X-chromosomal gebundene Erbgang der Hämophilie A ist seit langem bekannt und gilt als eines ihrer zuverlässigsten Merkmale. Wenn man also im Idiogramm nach einer morphologischen Manifestation der Krankheit fahndet, wird man vor allem das X-Chromosom untersuchen müssen. 1962 berichten Kosenow und Pfeiffer (7), bei der Hämophilie seien keinerlei Chromosomenabnormitäten gefunden worden. Im gleichen Jahr beschrieben Elves und Israels (3) ein abnormal langes Chromosom bei einem Fall von Hämophilie, das von den Verfassern als X-Chromosom angesehen wurde. Allerdings wies der betreffende Patient außer dem Blutungsübel noch weitere kongenitale Mißbildungen auf. Es lag also nahe, die gefundene Chromosomenaberration in erster Linie für die Malformationen verantwortlich zu machen und die Hämophilie nur in indirektem Zusammenhang damit oder überhaupt isoliert davon zu sehen. Wir hielten es daher für angebracht, die Chromosomen der von uns beobachteten Familie mit Hämophilie, deren Mitglieder keinerlei andere Mißbildungen erkennen lassen, zu untersuchen. Da Form und Länge des von Elves und Israels gefundenen Chromosoms genau mit den unseren übereinstimmen, schließen wir bei dem X-Chromosom mit verlängertem Arm auf einen ursächlichen Zusammenhang mit der Hämophilie. Zur Klärung der Entstehung dieser Anomalie werden noch weitere Merkmalsträger untersucht werden müssen. Wahrscheinlich liegt eine während der Oogenesis bzw. Spermiogenesis entstandene Translokation oder Reduplikation eines Chromosomenabschnittes vor. Die Schleimhautabstriche der Familienangehörigen unseres Bluters entsprachen dem Geschlecht, d. h. nur bei den Konduktorinnen waren Barrkörper vorhanden.

Von genetischem Interesse ist weiterhin die Frage, warum trotz des rezessiven Erbgangs bei den Konduktorinnen, die im heterozygoten Zustand theoretisch hinsichtlich der Hämophilie vollkommen merkmalsfrei sein müßten, Blutgerinnungsanomalien im Sinne einer unterschwelligen Hämophilie feststellbar sind. Es wird in diesem Zusammenhang von einem semidominanten Erbgang oder von einer unvollständigen Penetranz des Hämophiliegens gesprochen (16). Eine Erklärung mag in der besonderen Stellung des X-Chromosoms im weiblichen Chromosomensatz zu finden

sein Bei einem homozygoten Individuum summieren sich die Wirkungen zweier alleler Gene eines Chromosomenpaares (sog Dosiseffekt) Da nun die Frau zwei, der Mann aber nur ein X Chromosom besitzt, müßten bei der Frau alle Wirkprodukte der X-chromosomal gebundenen Gene doppelt so stark vorhanden sein wie beim Mann Das ist wie in letzter Zeit wiederholt exakt bewiesen wurde – z. B durch BEUTLER et al. (1) anhand der Glucose-6-Phosphat Dehydrogenase-Aktivität – mit wenigen Ausnahmen nicht der Fall. Eine Erklärung hierfür bietet sich in der 1961 aufgestellten «LYON Hypothese» (10) an. Danach bleibt in den somatischen Zellen der Frau ein X Chromosom genisch inaktiv entspiralisiert sich in der Telophase nicht sondern bleibt in der Interphase als Barrkörper an der Peripherie des Kernes liegen Das funktionstüchtige Chromosom kann entweder das von der Mutter oder vom Vater ererbte sein Offenbar ist die Verteilung der mütterlichen bzw väterlichen aktiven X Chromosomen im weiblichen Körper dem Zufall überlassen, so daß wir ein Mosaik vor uns haben in dem jeweils 50 / der Zellen ein von dem einen Elter ererbtes genetisch aktives X Chromosom besitzen. Inzwischen hat sich gezeigt daß die LYON-Hypothese nicht in jedem Fall zutrifft (17) Allein die Tatsache daß zwischen einer Frau mit der Chromosomenkonstitution X0 (Turner-Syndrom) und einer normalen Frau mit XX erhebliche Unterschiede bestehen, sowie die Auffindung der X-chromosomal gebundenen Blutgruppe Xg durch MANN et al (11) sprechen für eine zumindest teilweise Aktivität auch des zweiten X-Chromosoms in der weiblichen Körperzelle. Offenbar bildet also nur ein Teil dieses X Chromosoms, und zwar in erster Linie der längere Arm den Barrkörper der Rest bleibt genisch aktiv Das hämophiliesteuernde Gen scheint auf Grund der gefundenen morphologischen Chromosomenabnormität auf dem Arm lokalisiert zu sein, der in der Interphase bei der weiblichen Körperzelle inaktiviert werden kann Bei einer im Hinblick auf die Hämophilie heterozygoten Konduktorin finden sich demnach 50 der Körperzellen in einem Zustand, in dem das rezessive Gen des aktiven X-Chromosoms wirksam werden kann, während das entsprechende dominante Gen für «Nicht Hämophilie» auf dem zweiten X-Chromosom inaktiv bleiben muß Damit erklärt sich die Stellung der Konduktorin im Hinblick auf die Manifestation der Hämophilie Das rezessive Gen auf dem X-Chromosom kann im heterozygoten Zustand phänotypisch bis zu einem gewissen Grad

manifest werden, weil das entsprechende dominante Allel in etwa der Hälfte der Körperzellen inaktiv bleibt.

Mit unserem Befund eines abnormalen X-Chromosoms bei Konduktorinnen könnte für die Zukunft ein neuer Weg zur Verifizierung dieses Personenkreises aufgezeigt werden, der die biochemischen Methoden ergänzt und bestätigt.

Zusammenfassung

Bei einem Hämophilie-A-Patienten wurde in der akuten Blutungsphase eine Ahaptoglobinämie festgestellt, die als temporär und nicht genetisch bedingt angesehen werden muß. Die Chromosomenanalysen dieses Patienten und die seiner Mutter und Schwester, die als Konduktorinnen ermittelt wurden, wiesen ein in seinem langen Arm vergrößertes X-Chromosom auf. Eine Erklärung der intermediären Stellung der Frau im Hinblick auf den erblichen Faktor-VIII-Mangel wird nach der Lyon-Hypothese gegeben, wonach in weiblichen Zellen jeweils ein X-Chromosom genetisch inaktiv bleibt.

Summary

In a patient with haemophilia A, ahaptoglobinaemia was seen during the acute haemorrhagic stage. It must be considered a temporary phenomenon and not genetically determined. Chromosome analyses in the patient, his mother and sister, who were found to be carriers, revealed an X-chromosome with an enlarged long arm. An explanation of the intermediary position of the woman in regard to hereditary factor VIII deficiency is given in terms of the lyonisation theory, according to which there is in female cells a genetically inactive X-chromosome.

Résumé

Un malade, ayant une hémophilie A, présenta durant la phase aiguë d'une hémorragie une ahaptoglobinémie qui doit être considérée comme temporaire et non pas comme génétiquement déterminée. L'analyse des chromosomes de ce malade, de sa mère et de sa sœur, qui furent reconnues comme conductrices, démontra un chromosome dont le grand bras était agrandi. Une explication de la position intermédiaire de la femme quant au manque héréditaire du facteur VIII est fournie par l'hypothèse de Lyon selon laquelle le chromosome X resterait génétiquement inactif dans les cellules femelles.

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In vitro and Local Coagulation of Heparinized and Citrated Blood from Skin Wounds*

By C. P. Dietrich, W. O. Cruz, J. R. Magalhães and J. Borensztajn

Normal bleeding time has been observed in two groups of experiments where blood taken from large vessels was incoagulable (a) during perfusion of dog hind leg preparation with citrated and deplatelized blood (1, 2) and (b) in 63% of dogs intravenously heparinized with 300 IU of heparin per kg body weight (3). These results are difficult to interpret if it is accepted that coagulation and platelet plug are the primary factors in bleeding arrest.

In order to examine whether or not blood oozing from skin cuts (as in the above experiments) remains as incoagulable as blood stemming from large vessels, a number of experiments were performed to test the ability of oozing blood to clot in capillary tubes. These surprisingly showed that, in the majority of cases, oozing blood is quite capable of clotting when allowed contact with glass despite the fact that, if left in contract with the tissues, it remains perfectly fluid for more than 30 minutes, in spite of a normal bleeding arrest in a control wound.

Methods

52 adult mongrel dogs of both sexes were used. Anesthesia, when necessary, was induced with sodium pentobarbital.

Determination of bleeding and clotting times: Bleeding time was defined as the time required to arrest skin haemorrhage in wounds (of about 10 mm long and 1 mm deep and inflicted with a safety razor blade) from which the blood was completely removed with filter paper at the end of each minute.

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At fixed intervals, generally t the end of the 1st, 3rd, and 5th minutes the accumulated blood was collected from the wound in a capillary tube (0.2–0.4 mm internal diameter) and the time it took to coagulate was calculated by breaking off small segments of the glass tube at 1 minute intervals. Immediately after each capillary tube collection the drying of the wound was completed with a filter paper. It should be recorded, also, that in some instances, the volume of blood oozing from the wound was too small for the clotting time to be dtermined. Controls were collected from jugular vein, using the same procedure.

Heparin administration. After the bleeding and clotting time had been calculated, 100–600 IU/kg body weight of heparin (Liquemine Roche, 5000 IU/ml) were injected intravenously and the determinations were repeated on a fresh batch of wounds inflicted 10 minutes after the heparin injection. I some of the experiments the heparin dose was repeated at half hour intervals until the bleeding time exceeded 30 minutes. Tests were then performed at 15 minutes intervals until the normal bleeding time was restored.

It has been shown (3) that dogs vary in their response to standard 300 IU/kg dose of heparin 63 % have a bleeding time of less than 12 minutes (heparin resistant types) 17% (heparin intermediate types) bleed for 15–30 minutes whilst the remaining 18% (heparin labile types) bleed for longer than 30 minutes. In the present investigation, representatives of all three types were employed.

Isolated hind leg preparation. The technique employed was that described (1) for the in vivo study of haemostasis, with some minor improvements. During perfusion with citrated arterial or citrated venous blood re-oxygenated in vitro (4) the test for bleeding and clotting times described above were performed.

Results

Blood collected during the first minute from dogs intravenously injected with 300 IU/kg of heparin, almost invariably failed to clot (table I) Blood collected from the second minute onwards, however always clotted when the bleeding time was less than 30 minutes. This contrasted sharply with the incoagulability of blood samples taken simultaneously from the jugular vein. With different doses of heparin the blood collected at the 5th minute also clotted, except when bleeding time after heparin injection exceeded 30 minutes (table I)

Similar results were obtained with the hind leg preparation (table II) Thus, during perfusion with arterial citrated blood, the bleeding time remained normal and blood collected at different times clotted in 28 cases out of 36. In preparations with a higher than normal bleeding time the blood clotted in 4 determinations out of 10 Where the bleeding time exceeded 30 minutes, however the blood failed to clot in 15 cases out of 19 Perfusion of the hind leg with citrated venous blood re-oxygenated in vitro always produced a bleeding time greater than 30 minutes and there was no coagulation of blood collected at different intervals. The relation-

Table I

Relation between bleeding time and coagulation time in blood taken from the skin wound in dogs injected with different doses of heparin. Blood coagulation in samples from jugular-vein after heparinization were always greater than 30 minutes. Results in brackets refer to one experiment. Bleeding time before heparin administration 4 to 9 minutes.

Heparine Dosage I.U./kg	Number of determinations	Bleeding times after heparin (minutes)	Clotting time in minutes in blood collected from the skin wound at different intervals — Time intervals, minutes: 1	2nd–3rd	5th	10th–15th	20th–25th
	7	6–12	>30 (14)	7 10			
	3	5– 7	>30	>30	>30		
100–300	22	10–29	>30	6–14	2 7	5–15	
	11	>30	>30	>30	>30	>30	>30
350–600	6	10–19			5–17		
	5	>30			>30		
650–900	3	>30			>30		

Table II

Relation between bleeding time and clotting time in blood taken from skin wounds in the hind leg preparation. Blood coagulation in samples taken from the arterial inlet and venous outlet were always greater than 30 minutes.

Type of blood perfusion	Bleeding time (minutes)	Number of determinations	Clotting time in minutes in blood collected from the skin wound at different intervals — Time intervals, minutes: 1st	3rd	5th	10th–15th	20th–25th
	3–10	32	2 10	2 13	1 12		
	7 11	6	>30	5– 7	3– 5		
	6–12	8	>30	>30	>30		
Arterial	13–21	4	6–15	5–15	4– 9	5	
	13–19	6	>30	>30	>30	>30	
	>30	4	17–30	10–30	7 15	5–30	
	>30	15	>30	>30	>30	>30	>30
Venous (reoxygenated)	>30	10	>30	>30	>30	>30	>30

ship between the arrest of bleeding and the ocurrence of coagulation in oozing blood was studied by both methods (tables I–II) in 68 out of [redacted] observations, arrest of bleeding was also accompanied by blood clotting whereas in 54 instances out of 58 coagulation failed to occur when the bleeding time exceeded 30 minutes.

In some preliminary experiments involving 12 dogs and some 100 tests, the blood was allowed to accumulate in the wound whose depth was just sufficient to accommodate the oozing volume without involving overflowing After an interval of 6–25 minutes a pellicle was formed, at the blood-air interface, which became progressively thicker and could be demonstrated by touching the blood surface with a fine needle or wire. On removal, this pellicle adhered to the edges of the wound and it could be observed that the blood immediately beneath it was perfectly fluid. The latter when collected in a glass capillary clotted in the normal time. The experiments were repeated with heparinized heparin resistant dogs and the results were identical provided exactly comparable wounds were made.

Discussion

The present investigation establishes three important facts

1 Blood collected from a skin wound in a glass capillary tube is able to clot both when the blood perfusing the region is citrated and when the whole dog is heparinized.

2. Clotting occurs only in blood collected after the second minute in heparinized dogs.

3 Blood left untouched in a wound does not clot but remains fluid long after the time required for bleeding arrest in a control test.

These findings suggest that the mechanism by means of which oozing blood clots is probably more important for the study of haemostasis than that pertaining to blood from great vessels. This was implicit in the work by Gibbs (5) and [redacted] clearly in accord with the results of Soulier (6) who found differences between the clotting of venous and the so called capillary blood in haemophilia and other haemorrhagic diseases.

The question of whether coagulation does or does not play a part in the mechanism of haemostasis seem still open. The fact that blood taken in a glass capillary tube clots whilst the same blood left untouched in the wound does not in no way allows one to

deduce what is actually occuring within the injured vessels since if the capillary tube evidence supports the idea of coagulation the situation in the untouched wound does not. Furthermore despite certain histological studies, Apitz (7) and Zucker (8) no convincing experimental demonstration is available to show that haemorrhage of minute vessels is arrested by the coagulation of blood inside the injured vessels.

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Summary

1 Citrated blood perfusing a dog hind leg preparation as well as heparinized blood in the whole animal, has the capacity to clot in glass capillary tubes when collected from skin wounds, in spite of its incoagulability when withdrawn from large vessels. This phenomenon is generally observed after the first minute of bleeding

2. Blood left untouched in a wound does not form a massive clot but only a thin pellicle at the blood air interface. The pellicle becomes progressively thicker but the blood below remains uniformly fluid. The time taken for the formation of this pellicle is twice to three times as long as that required for bleeding arrest.

Résumé

Le sang citraté qui perfuse une préparation de l'extrémité arrière d un chien et le sang héparinisé d'un animal intacte ont la faculté de se coaguler dans un tube capillaire de verre s'ils ont été récoltés de blessures cutanées, mais ils restent incoagulables s'ils sont retirés de vaisseaux d un gros calibre Ce phénomène est observé généralement après la première minute de saignement. Si le sang est laissé intacte dans la blessure il ne se forme pas un caillot massif mais uniquement une pellicule mince entre le sang et l'air Cette pellicule s'épaissit peu à peu, mais en dessous le sang reste uniformément fluide. Le temps nécessaire à la formation de cette pellicule est deux à trois fois aussi long que celui requis pour l'arrêt d'un saignement.

Zusammenfassung

Zitratblut, das ein Präparat einer hinteren Extremität des Hundes durchströmt, und heparinisiertes Blut im intakten Tier besitzen die Fähigkeit, in Glaskapillaren zu gerinnen, wenn sie aus Hautwunden gewonnen werden, während sie bei Entnahme aus grossen Gefäßen ungerinnbar sind. Dieses Phänomen wird im allgemeinen nach der ersten Minute der Blutung beobachtet. Blut, das unberührt in einer Wunde belassen wird, bildet kein massives Gerinnsel, sondern nur eine dünne Haut an der Grenze zwischen Blut und Luft Diese Haut wird zunehmend dicker aber darunter bleibt das Blut in seiner Gesamtheit flüssig Die Bildung dieser Haut beansprucht zwei- bis dreimal soviel Zeit als die Blutstillung.

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XIIIth Colloquium, Bruges April 29–Mai 2, 1965

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Libri

S. Barandun: Die Gammaglobulin-Therapie. Chemische, immunologische und klinische Grundlagen. Bibliotheca Haematologica, Fasc. 17. S. Karger AG, Basel/New York 1964. VI + 138 S., 12 Abb., 26 Tab., Preis sFr. / DM 32.

Die Arbeitsgruppe für Eiweißfraktionierung am Zentrallaboratorium vom Schweizerischen Roten Kreuz in Bern ist seit über zehn Jahren an führender Stelle bei der Aufklärung der Gammaglobulinfraktion des menschlichen Blutserums tätig. Die klinischen und bakteriologischen Gesichtspunkte wurden in sehr aktiver Weise von Barandun bearbeitet. In der vorliegenden Monographie sind die Erfahrungen in vorbildlich klarer und übersichtlicher Weise zusammengestellt. Besonders bearbeitet wurden Voraussetzungen und Schwierigkeiten, Vorteile und Gefahren der intravenösen Applikation von Gammaglobulin. Seit Einführung von Gammaglobulin-Präparaten in die Therapie war bekannt, daß manche Menschen («Reagenten»), zu denen vor allem die Patienten mit Hypogammaglobulinämie gehören, auf die intravenöse Applikation von Gammaglobulin mit schweren schockähnlichen Zuständen reagieren. Es ist ein besonderes Verdienst der Berner Studiengruppe, die Natur dieses Zustandes mit großer Wahrscheinlichkeit als antikomplementäre Reaktion aufgeklärt zu haben. Die zahlreichen Untersuchungen, die zu diesem Ergebnis führten, werden ausführlich geschildert. Das nächste Anliegen Baranduns war die Unterdrückung dieser Reaktionen. Dieses Ziel ist heute theoretisch annähernd erreicht, wenn auch die industrielle Herstellung größerer schockfreier Gammaglobulinmengen noch auf beträchtliche Schwierigkeiten stößt. Eine Methode ist der partielle tryptische Abbau von Gammaglobulin (verwirklicht im «Gamma Venin» der Behring-Werke); die Berner Studiengruppe wählte statt dessen die vorübergehende Senkung des pH auf einen Wert von 4, wodurch ebenfalls eine physiko-chemische Veränderung des Moleküls eintritt. Die Vor- und Nachteile der beiden Methoden werden besprochen. Die ausgedehnte klinische Erfahrung des Autors wird sorgfältig und unter Berücksichtigung aller denkbaren Gesichtspunkte ausgewertet. Er kann daraus eine Liste der Indikationen und Dosierungen für die Gammaglobulin-Therapie ableiten, die für den therapeutisch tätigen Arzt sehr wertvoll sein wird. Als einzige Kritik kann man einwenden, daß in der ganzen Monographie der Preis der Gammaglobulin-Therapie nicht erwähnt wird, der in den angegebenen Dosen in der Praxis häufig prohibitiv ist; es ist jedoch zu hoffen, daß in Zukunft

eine Preissenkung zu einer Änderung führen wird. Mit seinem gründlichen Studium aller mit dem Thema zusammenhängenden Probleme bis zu den letzten Konsequenzen hat der Autor Ärzten und Patienten auf lange Sicht einen unschätzbaren Dienst erwiesen. Die originelle und informative Monographie ist für alle Ärzte, die sich in irgendeiner Form mit Hämotherapie beschäftigen, ein unentbehrlicher Wegweiser.

W. Hitzig, Zürich

H. Willenegger, G. W. Orth und W. Spielmann. **Ergebnisse der Bluttransfusionsforschung.** Band VIII. Bibliotheca Haematologica, Fasc. 20, S. Karger AG, Basel/New York 1964. VI + 318 S., 89 Abb., 44 Tab., Preis sF./DM 45.–

Der vorliegende 20. Band der Bibliotheca Haematologica enthält die anläßlich der 11. Tagung der Deutschen Gesellschaft für Bluttransfusion in Bad Nauheim gehaltenen Vorträge und Podiumsdiskussionen. Diese gruppieren sich um folgende Hauptthemen: Der hämolytische Transfusionszwischenfall; Indikation der Bluttransfusion; Therapie mit Blutbestandteilen und Morbus haemolyticus neonatorum.

Wie gewohnt übermittelt dieser Kongreßbericht eine ausgezeichnete Übersicht über einige aktuelle Probleme des Transfusionswesens.

A. Hässig, Bern

Iron Metabolism. An International Symposium. Sponsored by Ciba, Aix-en-Provence 1963, Springer Berlin/Göttingen/Heidelberg 1964. XII + 629 Seiten, 234 Abb., Preis DM 45.–

Die bedeutendsten Forscher aus aller Welt haben an dem von der CIBA Basel in Aix-en-Provence organisierten Symposium über Eisenstoffwechsel ihre neuesten Ergebnisse mitgeteilt. Die Beiträge betreffen die Biochemie des Eisens, des Ferritins und des Hämosiderins, die Physiologie des Eisens und des Transferrins, den Eisenmangel, die Eisenspeicherkrankheiten und die Behandlung der Eisenstoffwechselstörungen.

Besondere Beachtung wird der Biochemie und der therapeutischen Anwendung des neu entdeckten eisenbindenden Desferrioxamins geschenkt. Diese Art Zusammenstellung der neuesten Ergebnisse auf dem rasch fortschreitenden Gebiete des Eisenstoffwechsels entspricht einem wahren Bedürfnis. Grundwissenschaftler und Kliniker können sich darin über alle diesbezüglichen Aspekte rasch und genau orientieren.

P. Frick, Zürich

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Anläßlich des Lissaboner Kongresses der europäischen Gesellschaft für Hämatologie veranstaltete die Internationale Gesellschaft für Bluttransfusion ein Symposium über die immunologischen und chemischen Eigenschaften der Blutgruppenantikörper. In 22 Kurzvorträgen berichteten 44 Forscher aus acht Ländern über ihre Ergebnisse. Das Heft vermittelt eine ausgezeichnete Übersicht über ein aktuelles, in raschem Fluß befindliches Forschungsgebiet.

A. Hässig, Bern

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Clinique Médicale Universitaire de Lausanne (Directeur: Prof. A. Vannotti)

L'exploration isotopique du métabolisme du fer

Anciens et nouveaux modèles

Par F. Clément, B. Delaloye et A. Vannotti

La technique et les modes de calcul mis au point par Finch (6) en 1949 puis surtout par Huff (10, 9) en 1950 sont couramment utilisés par les auteurs qui se livrent à l'exploration du métabolisme du fer au moyen des isotopes radioactifs. Sans revenir sur les détails, nous ne voulons ici que rappeler brièvement les principaux concepts auxquels il est classique de faire appel dans ces travaux.

Au cours des heures qui suivent l'injection du fer marqué (lié à la sidérophiline) on observe une disparition de la radioactivité du plasma. Les valeurs enregistrées permettent de tracer une courbe exponentielle; la pente de la droite que l'on obtient en portant cette exponentielle sur un papier semi-logarithmique traduit la vitesse à laquelle le fer quitte le plasma et donne par conséquent une idée de l'«avidité» de la moelle pour le fer. La mesure de la période (T½) de cette décroissance, ainsi que du volume plasmatique (que l'on déduit de la radioactivité du plasma au temps 0, obtenue par extrapolation) permet, la valeur du fer sérique étant connue, de calculer la *vitesse de renouvellement du fer plasmatique*, c'est-à-dire la quantité de fer du plasma qui est renouvelée en 24 heures. Connaissant

(FS) = le taux du fer sérique en mg/ml

$$(VP) = \frac{\text{radioactivité totale injectée}}{\text{radioactivité de 1 ml de plasma à } t_0} = \text{le volume plasmatique en ml}$$

T½ = période de décroissance du fer plasmatique en heures,

on obtient le taux de renouvellement du fer plasmatique selon la formule

$$\frac{0.693}{T½} \cdot (FS) \cdot (VP) \cdot 24 = \text{mg/jour}$$

Normalement cette vitesse est de 30–35 mg/jour, soit 0.45 à 0.65 mg/kg/jour. Cette valeur exprime en principe l'importance quantitative de l'érythropoïèse; on la trouvera donc augmentée dans les anémies hypochromes par carence en fer, dans les anémies hémolytiques, l'anémie de Biermer et les polyglobulies; elle sera diminuée, c'est-à-dire que la pente sera plus faible que normalement, dans les anémies aplastiques.

Le coefficient d'incorporation globulaire, c'est-à-dire la fraction de la radioactivité injectée que l'on retrouve dans la circulation lorsque le plateau est atteint, généralement vers le 10me jour, reflète, lui, l'efficacité de l'érythropoïèse. Il est normalement de 70 à 90%; on le trouve augmenté dans les anémies par carence, diminué dans les anémies

hémolytiques, les anémies infectieuses, l'hémochromatose et surtout les anémies aplastiques. Soit

(VS) = le volume sanguin en ml (facile à obtenir à partir du volume plasmatique et de l'hématocrite, corrigé par le coefficient 0.87)

le coefficient d'incorporation sera donné par la formule:

$$\frac{\text{radioactivité/ml (VS) 100}}{\text{radioactivité totale injectée}} = x\%$$

La vitesse d'utilisation, c'est-à-dire le temps mis par l'organisme pour incorporer dans les érythrocytes circulants la moitié de ce qu'il est capable d'incorporer, est normalement de 4 jours. Ce temps est généralement abrégé lorsque l'incorporation totale est augmentée et allongé dans le cas contraire; en revanche lors d'hémolyse on trouve une vitesse d'utilisation accélérée malgré une incorporation diminuée, la fin de la courbe d'incorporation étant amputée par le processus hémolytique lui-même. La vitesse d'utilisation est aussi accélérée lorsqu'il y a sortie prématurée des globules rouges hors des centres hématopoïétiques comme c'est peut-être le cas dans la splénomégalie myéloïde (4).

La vitesse de renouvellement du fer globulaire est égale au produit de la vitesse de renouvellement du fer plasmatique par le coefficient d'incorporation, c'est-à-dire la fraction du fer qui est destinée aux hématies. Sa valeur normale est de 0.35 à 0.55 mg/kg/jour. Le rapport du fer globulaire ainsi renouvelé chaque jour sur le fer globulaire circulant total, devrait en principe permettre de calculer la durée de vie moyenne des érythrocytes.

Vitesse de renouvellement du fer globulaire

$$\text{Renouvellement du fer plasmatique} \cdot \frac{\text{Coefficient d'incorporation}}{100} = x \text{ mg/jour}$$

$$\text{soit (FGT)} = \frac{(VS)}{100} \cdot \text{Hb. en g/100 ml} \cdot 3.34 = \text{fer glob. total en mg.}$$

la quantité de fer globulaire renouvelée par jour sera

$$\frac{\text{Renouvellement du fer globulaire}}{\text{(FGT)}} = X\%$$

$$\text{d'où Durée de vie érythrocytaire calculée } \frac{100}{x\%} \text{ jours.}$$

Les comptages externes sont effectués sur la moelle (sternum et sacrum), le foie et la rate. Ils permettent une évaluation très approximative mais fort précieuse des mouvements du fer dans ces organes. Il est indispensable de ne tracer ces courbes qu'après avoir corrigé les valeurs brutes obtenues en fonction de la radioactivité circulante, selon la technique de HUFF (9) et BUSH (3) si l'on ne veut pas ajouter à une évaluation déjà grossière, par le fait des variations dans les conditions de géométrie, une autre variable qui, elle, est facilement éliminable. Rappelons le principe de cette correction: à partir de la courbe de décroissance plasmatique et des comptages externes effectués lors des premières minutes de l'épreuve, il est possible de calculer par extrapolation la radioactivité externe de chaque organe à t_0, radioactivité ne dépendant en principe que de la vascularisation de cet organe. La connaissance de l'incorporation globulaire à chaque jour de l'épreuve permet alors ensuite de trouver la radioactivité de chaque organe pour chaque mesure successive, qui est théoriquement imputable aux globules circulants et de la déduire de la radioactivité brute enregistrée. En outre on exprimera les résultats après les avoir divisés par la radioactivité injectée afin de pouvoir comparer l'amplitude de ces courbes d'un sujet à l'autre. Normalement, les courbes enregistrées sur les régions médullaires montrent une ascension qui atteint le maximum dès le premier jour, suivie d'une dé-

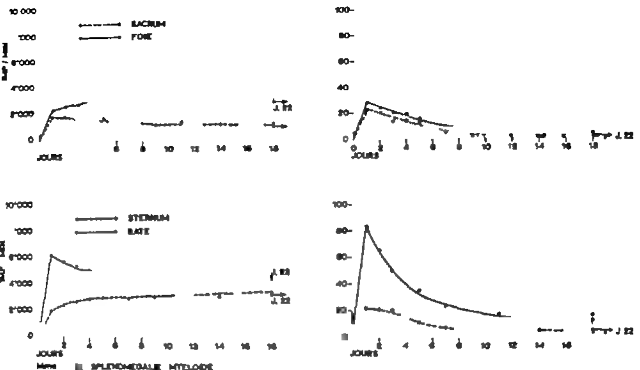


Fig. 1. Comptages externes sans et avec correction en fonction de la radioactivité circulante. D. Livette, 58 ans, splénomégalie myéloïde. Les courbes non corrigées à gauche permettent de voir que la rate est le siège d'une légère libération érythrocytaire mais non de se rendre compte de l'importance de ce phénomène; ainsi il est impossible d'après cette courbe de dire s'il existe une éventuelle érythrolyse splénique surajoutée comme cela s'observe fréquemment dans la splénomégalie myéloïde, alors que la courbe corrigée à droite montre clairement qu'il n'y en a pas. De même la discrète érythroformation hépatique, visible sur la courbe corrigée, est peu apparente sur la courbe brutes.

cente, rapide elle aussi, qui correspond à la libération érythrocytaire; les courbes hépatique et splénique ont une amplitude environ deux fois plus faible. Lorsque la moelle est insuffisante, on verra une courbe externe qui s'élève peu et qui ne redescend que très lentement; les courbes splénique et surtout hépatique permettent de se faire une idée de l'importance des réserves: on observera alors un tracé hépatique montant rapidement à une valeur beaucoup plus élevée que le pic de l'activité sur le sacrum puis suivie d'un plateau; il en est ainsi par exemple dans l'hémochromatose; lorsque le fer ne va pas dans la moelle il lui faut se fixer davantage dans le foie et la rate sans que pour cela les réserves soient forcément augmentées; lors d'hémolyse, on pourra voir une activité splénique augmentant progressivement au cours de l'épreuve. Enfin dans les cas de splénomégalie myéloïde, le comptage externe pourra être très instructif en mettant en évidence, à côté de l'insuffisance de la moelle, une bonne fixation de l'isotope dans la rate et éventuellement le foie suivie d'une libération rapide de type médullaire, dans la mesure tout au moins où une hémolyse surajoutée ne vient pas ralentir cette descente (4) (fig. 1).

Cette interprétation «classique» des mouvements du fer repose sur la conception que l'essentiel du fer de l'organisme se situe dans deux pools, l'un plasmatique, l'autre érythroblastique-érythrocytaire. Cependant, l'étude des courbes expérimentales montre qu'il n'est pas possible de tout expliquer par un schéma aussi simple. Il arrive souvent que l'on trouve un renouvellement plasmatique élevé et une incorporation globulaire basse, sans hémolyse notable. L'interprétation habituelle d'une telle conjoncture est que l'érythropoïèse est quantitativement augmentée mais qualitativement insuffisante, c'est-à-dire inefficace. Un tel tableau se rencontre dans l'anémie de BIERMER, dans la thalassémie (12) et autres anémies hypochromes hypersidérémiques, dans le syndrome de FANCONI, dans l'hémochromatose (où l'on invoque plutôt la dilution extrême de l'isotope) et dans de nombreux cas de pancytopénie chronique idiopathique. On tente de l'expliquer soit par un ralentissement de la maturation érythroblastique ou de la libération réticulocytaire soit par un trouble de l'incorporation du fer dans l'hémoglobine (explication cependant non valable dans la mesure où les sidérocytes peuvent sortir de la moelle) soit encore par la destruction intra-médullaire d'une fraction érythroblastique ou érythrocytaire: ce dernier mécanisme mérite d'être retenu car il existe certainement, en particulier dans la thalassémie et l'anémie de Biermer. Cependant l'accélération excessive du renouvellement plasmatique ne se rencontre pas que dans ces dernières affections: au contraire, il est extrêmement fréquent et il est parfois d'une importance manifestement absurde, en particulier dans la polycythémie (21, 8). Plus encore, le renouvellement plasmatique paraît trop élevé même chez le sujet normal, car lorsque l'on s'en sert pour calculer le renouvellement globulaire, on aboutit à une

estimation de la durée de vie moyenne érythrocytaire qui est trop courte (jusqu'à 50 jours selon certains, 4). Il était donc sensé d'imaginer que tout le fer qui sort du plasma n'entre pas directement dans l'érythroblaste mais qu'il s'arrête dans un 3e pool du fer d'où une partie entrera définitivement dans l'érythroblaste et l'autre partie reviendra vers le plasma. En outre, l'analyse des courbes expérimentales, lorsqu'on prend soin de mesurer la radioactivité plasmatique pendant plusieurs jours et non pas seulement une ou deux heures après l'injection de l'isotope, révèle l'existence d'une seconde courbe de décroissance plasmatique exponentielle de période beaucoup plus longue qui se manifeste généralement dès les premières 24 h. Cette seconde exponentielle suggère la réalimentation en fer du plasma à partir d'un 3e pool du fer distinct du pool globulaire. Enfin l'incorporation complète de l'isotope n'est atteinte qu'après 10 à 12 jours, alors que l'on sait que la maturation érythroblastique et le séjour intra médullaire des réticulocytes ne dépasse pas 4 à 5 jours (1): cette discordance suggère donc elle aussi l'existence d'un pool interposé. En 1955 déjà, SHARNEY et coll. (21) ont conçu un modèle de 3 pools dont le 3e, mobile, peut livrer son fer tant au second qu'au premier: ils ont proposé une formulation mathématique permettant de calculer l'importance respective de chacun de ces pools. NAJEAN (14), qui a développé ces problèmes dans sa thèse, estime aussi qu'avant de juger de l'importance quantitative de l'érythropoïèse, il faut corriger le taux du renouvellement plasmatique en fonction de cette formule. Il obtient ainsi des valeurs qui sont souvent d'environ 30% (de 15% à 60%) plus faibles et paraissent donc plus près de la réalité. Il est à noter que l'étude mathématique a montré que l'hypothèse de la destruction précoce d'une population d'hématies ne permettait pas d'expliquer la présence de la seconde exponentielle.

En 1961 POLLYCOVE ET MORTIMER (20) ont proposé un nouveau modèle qui devrait refléter mieux encore la réalité des mouvements du fer dans l'organisme: ce modèle, qui donne toute son importance au 3e pool labile, permet aussi de chiffrer l'importance des réserves.

Le modèle de POLLYCOVE ET MORTIMER est composé de 6 pools (fig. 2)

1. le pool plasmatique, qui se déverse dans deux pools «labiles», c'est-à-dire aux échanges très faciles, qui sont:

2. le pool labile érythropoïétique, que l'on peut considérer comme l'antichambre du pool érythroblastique auquel il fournit son fer mais qui renvoie aussi une partie de ce

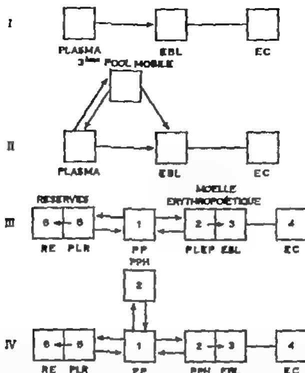


Fig. 2. I. Modèle «classique» II. Modèle selon SHARNEY (21). III. Modèle de POLLYCOVE (20). IV. Modèle de NAJEAN (16). EBL = Erythroblastes. EC = Erythrocytes. RE = Réserves «échangeables». PLR = Pool labile des réserves. PP = Pool plasmatique. PLEP = Pool labile érythropoïétique. PPH = Pool préhémique.

qu'il a accepté au pool plasmatique et, 5. le pool labile des réserves qui remplit les mêmes fonctions vis-à-vis des réserves et qui, lui aussi, renvoie une partie de son fer au pool plasmatique.

3. le pool érythroblastique, dont le fer ne peut alors plus sortir que pour passer dans 4. le pool érythrocytaire. Enfin 6. le pool du fer «échangeable» des réserves qui ne renvoie du fer plasma qu'à un débit extrêmement faible. Il est à noter que le pool labile érythropoïétique est physiologiquement présent (65 à 105 mg) alors que les pools 5 et 6 des réserves sont négligeables à l'état normal. Grâce à ce modèle, les auteurs calculent la synthèse quotidienne d'hémoglobine les valeurs trouvées semblent proches de la *réalité puisqu'il est possible d'en déduire une durée de vie moyenne de 110 à 125 jours;* dans l'hémochromatose ils trouvent ainsi une synthèse d'hémoglobine normale correspondant donc à une durée de vie moyenne normale et ils évaluent quantitativement l'importance des réserves du fer

Dans l'analyse graphique de leurs courbes expérimentales, ces auteurs considèrent qu'il existe deux exponentielles à l'état normal mais lorsque les réserves sont augmentées, ils en construisent trois, ce qui est un minimum au vu de la courbe obtenue et ce qui correspond à une réalimentation du plasma à partir de deux pools labiles. La 3e exponentielle traduit le reflux du fer du pool labile érythropoïétique vers le plasma; sa présence est constamment observée alors que la seconde exponentielle est pas décelable à l'état normal. 6 équations à 6 inconnues permettent à ces auteurs de calculer les débits de chacun de ces pools l'un vers l'autre et la valeur de chacun d'entre eux. Les différentes

interréactions qu'exercent ces mouvements permettent difficilement, à la seule inspection de la courbe expérimentale de juger avec précision de leur importance respective; cependant la pente de la première exponentielle est d'autant plus grande que le mouvement du fer du plasma vers le pool labile érythropoïétique est plus important quant à la 3e exponentielle, elle dépend essentiellement des mouvements du fer du pool labile érythropoïétique vers le pool érythroblastique. En outre, un grand pool labile érythropoïétique renverra son fer tardivement mais pendant longtemps, alors qu'un petit pool labile érythropoïétique le renverra précocement mais brièvement. Enfin à partir du 14e jour la radioactivité plasmatique cesse de décroître et reste constante ce qui est à mettre en relation avec le retour extrêmement lent du fer provenant des réserves relativement fixes dites «échangeables». L'importance de ce dernier pool sera proportionnelle à la quantité de fer non incorporé et inversément proportionnelle à la radioactivité résiduelle du plasma. En cas d'hémolyse, l'activité plasmatique s'équilibre plus précocément et il n'est alors plus possible de calculer le pool des réserves «échangeables» à moins d'avoir recours à une évaluation fondée sur l'importance de la radioactivité hépatique.

A quoi correspond ce pool labile érythropoïétique? Pour Pollycove et Mortimer il est constitué par le fer absorbé d'une manière réversible à la paroi de l'érythroblaste et pouvant soit être incorporé dans cette cellule soit être relâché dans le plasma. Pour Najean (15, 16, 2) par contre, il n'est pas possible de caractériser ce pool par une définition topographique (médullaire) il faut au contraire le considérer comme ubiquitaire, être mathématique plus qu'anatomique. Cet auteur avait déjà démontré théoriquement que l'observation de Pollycove, que le pool labile érythropoïétique est très petit dans les aplasies, ne prouve nullement que ce compartiment soit uniquement érythroblastique. Ses travaux sur l'incorporation du fer marqué dans le réticulocyte, in vitro (18, 19) semblent prouver que le compartiment, interposé entre le plasma et l'hème, présent dans le réticulocyte est d'une importance quantitative dérisoire par rapport à l'ensemble du pool «préhéminique» comme il l'appelle, tel qu'il est mesuré d'après les recherches effectuées in vivo. En d'autres termes, sur notre schéma des différents modèles, il adopte désormais le schéma IV et considère 2 négligeable par rapport à 2. Ce problème n'a d'ailleurs qu'une portée théorique, puisque, quel que soit le modèle utilisé (III ou IV) le calcul de la synthèse de l'hème aboutit au même résultat. Or c'est là que réside l'intérêt principal de ces nouveaux modèles.

Méthode

Depuis deux ans, nous interprétons les résultats fournis par l'exploration isotopique du métabolisme du fer en fonction des modèles proposés par Pollycove (20). Il est nécessaire alors de pratiquer des prélèvements du plasma pour comptages, non seulement pendant les 2 premières heures comme le veut l'épreuve classique, mais pendant les 6 ou 7 premières heures, puis à la 12e heure environ et enfin tous les jours, puis tous les deux ou

trois jours, pendant deux à quatre semaines. Pour le reste, la technique est semblable à celle exposée par Mercs-Berx et coll. (13) à cela près que nous n avons pas ajouté de sidérophiline étrangère à la préparation et que nous avons limité les comptages externes au sacrum, au sternum, au foie et à la rate. Les comptages des échantillons sanguins sont effectués au cristal creux activé au thallium, durant un laps de temps suffisant pour que l'erreur de mesure ne dépasse pas 2%. Nous utilisons un Fe^{59} de haute activité spécifique provenant de Saclay et de Oak Ridge.

Les informations que l'on peut ainsi espérer obtenir concernent d'une part la valeur de la synthèse réelle de l'Hb, d'autre part l'évaluation quantitative des mouvements vers les réserves.

Résultats

1 Utilisation réelle du fer pour la synthèse de l'hémoglobine

Nous avons pu l étudier en particulier dans un cas de myélofibrose avec hématopoïèse extramédullaire.

Il s'agit d'une femme de 58 ans, Madame D. Lisette. Cette patiente souffre de fatigue depuis de nombreuses années. Une splénomégalie et une anémie sont découvertes en 1858. A son admission en octobre 1963, la patiente présente une rate volumineuse touchant presque l'épine iliaque antéro-supérieure et une hépatomégalie modérée. Les formules sanguines montrent une anémie à 72% d'Hb vec micro- et poïkilocytose, une leucocytose entre 9 et 14,000/mm avec quelques érythroblastes et 6% de myélocytes. De nombreuses ponctions sternales restent infructueuses. La ponction de la rate ramène un matériel érythromyéloïde caractéristique de la myélofibrose. La biopsie osseuse révèle une moelle riche avec hyperplasie mégacaryocytaire, comme c'est bien souvent le cas dans les stades précoces de cette affection.

L épreuve au Fe^{59} confirme le diagnostic on voit nettement d après les courbes de comptages externes (fig 1) que la rate assure la fonction érythropoïétique gravement déficiente dans les territoires médullaires. L incorporation globulaire est à peine diminuée et la vitesse d utilisation est augmentée, probablement du fait d une sortie plus précoce des réticulocytes de la rate que de la moelle. Les mesures de la radioactivité plasmatique ont été effectuées jusqu au 22e jour (fig 3) il est aisé de déceler dans cette courbe un système de deux exponentielles. Le renouvellement plasmatique du fer se révèle augmenté malgré un fer sérique relativement bas cette observation, jointe à la constatation d une incorporation diminuée, serait considérée, selon les notions «classiques» comme le reflet d une érythropoïèse quantitativement augmentée mais qualitativement déficiente, en d autres termes, d une érythropoïèse dite «inefficace» Le renouvellement du fer globulaire, augmenté lui aussi, permet de calculer une synthèse quotidienne d Hb de 11,5 g/jour et par conséquent une durée de vie globulaire nettement raccourcie à 50 jours. Les calculs selon Pollycove (tableau I)

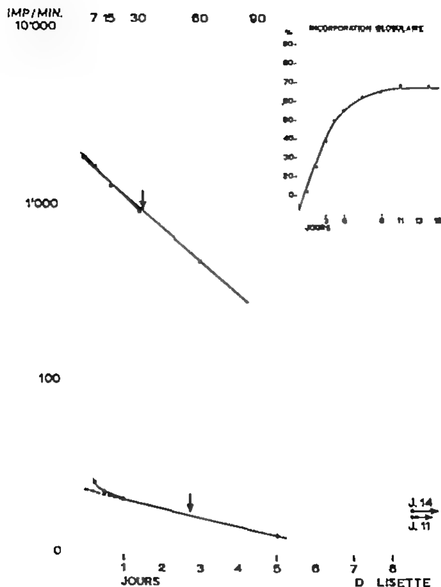


Fig. 3. D. Lisette, splénomégalie myéloïde. Décroissance plasmatique et incorporation globulaire.

Tableau I

D. Lisette Résultats des calculs «classiques» et selon le modèle de POLLYCOVE.

Fe⁵⁹ Nom D Lisette	Dg.: splénomégalie myéloïde		Normes	Modèle Pollycove			Normes
							Date: 11 nov 1963
Fer sérique	34	γ%		1re expon. %: ///	T½: 0.5 h		
Vol. plasmatique:	3,340	ml		2e expon. %: ///	T½: /// jours		
Vol. sanguin:	5,130	ml		3e expon. %: 0.012	T½ 2.75 jours		
Incorporation glob.:	63	%	70–90	X_1:		1.8 mg	3.6±0.7
Vitesse d utilisation:	2.3	jours	4	X_2 (Pool labile EP) $\alpha_{32}X_2$ (Mouvement fer vers Hb)		93 mg 23 mg/j	83±20 21.4±3.2
Modèle classique				Hb réellement synth./jour		6.8 g/j	6.3±0.9
Renouv. fer plasmatique:	0.95	mg/kg/j	0.45–0.63	Quant. Hb renouv./jour:		1.2 %	
Renouv. fer globulaire:	0.61 = 29 soit: 11.5	mg/kg/j mg/j g Hb/j	0.35–0.55 30–35	Durée vie glob. calculée:		86 jours	117±8
				X_4 (Pool labile réserves):		/// mg	
Quant. fer renouvelé/jour:	2	%		$\alpha_{42}X_2$ (Mouvement fer vers rés.)		/// mg/j	1
Durée vie glob. calculée:	50	jours		λ_{ex} (Fer échangeables)		Impossible à calculer	500

mettent en évidence un «pool labile érythropoïétique» ou «pool préhémique» selon la nomenclature que l'on choisit, de 93 mg c'est-à-dire normal les mêmes résultats ont été obtenus par NAJEAN (16) dans la splénomégalie myéloïde. Grâce à ce modèle, l'Hb réellement synthétisée se révèle n'être que de 6 8 g/jour valeur tout à fait normale et par conséquent la durée de vie globulaire atteint 86 jours, ce qui correspond à une hémolyse très modérée. Or le taux bas de la bilirubinémie (totale 0,3 mg%) et du fer sérique, la réticulocytose à peine élevée (100 000/mm³) l'absence de descente secondaire de la courbe d'incorporation et de remontée de la radioactivité sur la région splénique sont autant d'arguments qui font penser que l'hémolyse est très discrète chez cette malade le modèle de POLLYCOVE paraît donc dans ce cas le plus proche de la réalité cette patiente fabrique chaque jour une quantité d'Hb normale, insuffisante toutefois pour compenser une très légère hémolyse. Quant à la mesure de X_{em}, le fer «échangeable» elle n'est pas possible ici, en raison d'une légère remontée de la radioactivité plasmatique dès le 9e jour qui rend la valeur de l'activité résiduelle dans la phase d'équilibre indéterminable.

2 *Les mouvements du fer vers les réserves*

L'étude quantitative des réserves paraît plus délicate et ses résultats plus discutables. Elle a pu être effectuée dans deux cas d'hémochromatose.

a) *Hémochromatose primitive*

Monsieur J Joseph, 50 ans, souffre d'une hémochromatose diagnostiquée par ponction-biopsie du foie en 1959 Le foie n'est pas agrandi et les seuls signes biologiques sont un prédiabète et un fer sérique à 250γ% avec saturation complète de la sidérophiline les tests hépatiques sont normaux. Le patient a subi de nombreuses saignées et il est soumis à une cure continue de desferrioxamine (Poli. Med. Univers. Lausanne, Prof. Ed. Jéquier) Au moment de l'examen, l'Hb est à 96% et le nombre des érythrocytes à 4,800,000/mm³ La moelle est riche, érythroblastique (38%).

L'épreuve au Fe^{59} (fig 4) montre une forte activité de la moelle (la remontée de la courbe sternale est probablement due à la proximité du foie) Les courbes de radioactivité externe ont été tracées et corrigées selon un autre principe, un incident technique ne nous ayant pas permis de mesurer la radioactivité globulaire chez ce patient il s'agit de la méthode de POLLYCOVE (20) qui consiste à établir le rapport de la radioactivité mesurée au jour J sur la radio-

Date 8 juil.

Fe⁵⁹	Nom: J. Joseph	Dg: Hémochromatose
Fer sérique:	220	γ%
Vol. plasmatique:	2.450	ml
Vol. sanguin:	4.000	ml
Incorp. glob.:	75	%
Vitesse d'utilisation:		jours
Modèle classique		
Renouv. fer plasmatique	1.9	mg/kg/j
Renouv. fer globulaire	0.97	mg/kg/j
	= 62	mg/j
	soit 18.2	g Hb/j
Quant. fer renouvelé/jour		%
Durée vie glob. calculée		jours
Modèle Pollycove		
1re expon. %	0.957	T½ 1.25 h
2e expon. %	0.030	T½ 0.15 jours
3e expon. %	0.003	T½ 1.3 jours
X_1	6.2	mg
X_2 (Pool labile EP):	112	mg
$a_{12}X_1$ (Mouvement fer vers Hb)	49	mg/j
Hb réellement synth./jour	14.3	g/j
Quant. Hb renouv./jour		%
Durée vie glob. calculée		jours
X_3 (Pool labile réserve)	19.6	mg
$a_{13}X_1$ (Mouvement fer vers rés.)	18.7	mg/j
X_{ex} (Fer échangeable)	300–1.700	mg

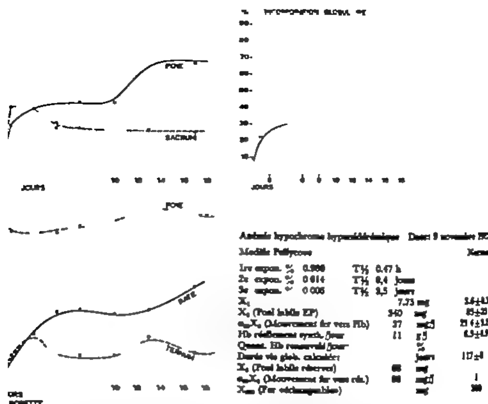
Fig. 4 J. Joseph, hémochromatose primitive. Décroissance plasmatique, courbes de radio-activité externe corrigées selon impulsion / minute / imp. / min₀. Résultats des calculs selon le modèle de POLLYCOVE.

activité au temps 0 (obtenue par extrapolation) de sorte que normalement toutes les courbes tendent à se rejoindre en 1 après quelques jours. La courbe hépatique met en évidence une mise en réserve certaine, du même ordre de grandeur que celle observée par cet auteur chez les patients atteints d'hémochromatose. Ne pouvant mesurer l'incorporation globulaire directement, nous avons recouru au procédé proposé par POLLYCOVE ET MORTIMER pour les cas où il existe une hémolyse : ces auteurs évaluent l'incorporation globulaire indirectement en se fondant sur la proportionalité qui existe entre l'augmentation de la radioactivité sur l'aire hépatique et la fraction du fer injecté qui n'est pas incorporée, le rapport entre ces deux paramètres étant de 2.6 environ. Nous avons décomposé ici la courbe de décroissance plasmatique en 3 exponentielles, la 3e étant obtenue après soustraction de la radioactivité due aux échanges lents, la seconde après soustraction de la 3e et de la radioactivité résiduelle. Les calculs selon les nouveaux modèles montrent alors un pool labile érythropoïétique très légèrement augmenté, un pool labile des réserves accru et un mouvement du fer vers les réserves égal à 17 fois la normale, résultats en tous points semblables à ceux des hémochromatoses de POLLYCOVE. Le fer «échangeable» ne peut pas être calculé avec précision, les comptages plasmatiques n'ayant pas été poursuivis suffisamment longtemps : cependant il ne semble pas être nettement augmenté, ce qui rappelle le cas d'hémochromatose débutante des mêmes auteurs. Quant à la synthèse «réelle» d'Hb elle paraît nettement surestimée ici (14.5 g/j) bien que ce résultat soit probablement déjà moins faux que celui fourni par le calcul «classique» de 18.2 g/j. Il n'est cependant peut-être pas complètement faux si l'on pense que ce patient a subi de très abondantes saignées et que sa moelle montre une hyperplasie érythropoïétique. En résumé, il semble donc s'agir d'une hémochromatose débutante comportant et un fort mouvement du fer vers les réserves et une importante activité médullaire.

b) Hémochromatose secondaire par anémie sidéroachrestique

L'interprétation des courbes se révèle particulièrement difficile chez Madame L. Rosette.

Cette patiente, âgée de 30 ans, présente le tableau d'une anémie hypochrome hypersidérémique, avec hémochromatose secondaire (confirmée par ponction biopsie du foie); la ponction médullaire a malheureusement toujours été refusée. La formule sanguine montre 52% d'Hb, 3,050,000 érythrocytes par mm³, V. G. 0.86. Fer sérique:



Anémie hypochrome hypersidérémique Date: 9 novembre 196[illegible]

Modèle Pollycove					Norm.
1re expon. %	0.980	T½	0.47 h		
2e expon. %	0.014	T½	0,4	jours	
3e expon. %	0.006	T½	3,5	jours	
X_1			7.73	mg	[illegible]
X_2 (Pool labile EP)			340	mg	[illegible]
$a_{32}X_2$ (Mouvement fer vers Hb)			37	mg/j	[illegible]
Hb réellement synth. /jour			11	g	[illegible]
Quant. Hb renouvelé /jour				%	
Durée vie glob. calculée				jours	117±[illegible]
X_4 (Pool labile réserves)			[illegible]	mg	
$a_{42}X_2$ (Mouvement fer vers rés.)			[illegible]	mg/j	1
X_{ex} (Fer échangeable)				mg	[illegible]

Fig. 5. L. Rosette hémochromatose secondaire à une anémie sidéroachrestique Courbes de radioactivité externe corrigées selon la radioactivité circulante et, au milieu, courbe de radioactivité externe hépatique corrigée selon imp. / min / imp. / min$_0$. Incorporation globulaire et résultats des calculs selon le modèle de Pollycove.

313γ%. Coefficient de saturation 0.91 (puis fer sérique 329γ%, coeff. de saturation 1.0). L'hépatosplénomégalie est importante.

L'incorporation globulaire du Fe^{59} (fig. 5) apparaît extrêmement basse (30 %) les courbes de comptages externes montrent une faible fixation médullaire avec sortie déficiente la mise en réserve hépato-splénique est nette. La solution des équations de Pollycove met en évidence un énorme pool labile érythropoïétique, un pool labile des réserves et un mouvement du fer vers les réserves très augmentés toutes ces valeurs sont bien supérieures à celles que nous avons relevées dans le cas précédent d'hémochromatose primitive et nous rejoignons en ceci les observations de Najean (16) que tant les compartiments labiles que le mouvement du fer vers les réserves sont beaucoup plus importants dans les anémies sidéroachrestiques que dans les hémochromatoses idiopathiques.

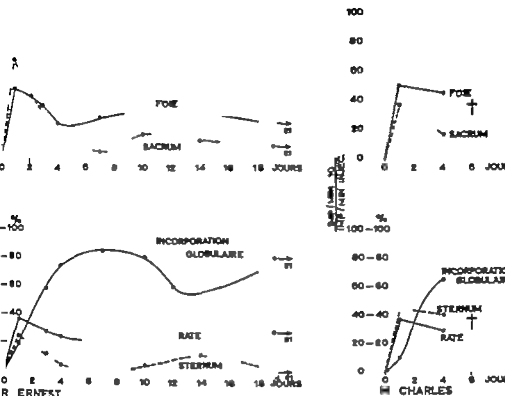


Fig. 6. R. Ernest et M. Charles, carcinomes bronchiques. Courbes de radioactivité externe, corrigées selon la radioactivité circulante et incorporations globulaires.

Si l'on calcule l'incorporation par la méthode indirecte de POLLYCOVE ET MORTIMER, à l'aide de la formule:

$$\frac{(\text{radioactivité hépatique max.} / \quad \text{a. hép. à } t_0) - 1}{1 - \text{incorporation}} = 2.6$$

on aboutit à une valeur de 90%. Est-ce à dire qu'il existe dans ce cas une hémolyse considérable? U certain degré d'hémolyse est très possible et même probable mais certainement pas d'une telle importance car la fixation médullaire est trop faible et la réticulocytose trop basse (36,000/mm³). Ce résultat paradoxal provient du fait que la méthode de correction de la radioactivité externe de POLLYCOVE (cpm/cpm_s) se trouve ici en défaut en effet, étant pas corrigées en fonction de l'incorporation globulaire, les valeurs des derniers jours se trouvent fortement sous-estimées; en d'autres termes, si l'incorporation était plus haute dans ce cas, pour une fixation hépatique «réelle» supposée identique, la valeur de cpm. max. hép. / cpm_0 hép., serait plus élevée de sorte que l'incorporation calculée serait plus petite!

3. *Métabolisme du fer dans l'anémie cancéreuse*

Nous avons soumis à l'exploration isotopique par le Fe^{59} trois patients atteints d'anémie cancéreuse sans spoliation sanguine ni hémolyse nette ni carcinose médullaire. L'anémie était chez tous modérée, deux fois normochrome, une fois hypochrome. La valeur du fer sérique se trouvait une fois légèrement augmentée, deux fois à la limite inférieure de la norme; dans ces deux derniers cas, la valeur absolue de la sidérémie (pool plasmatique) était nettement diminuée.

Monsieur R. Ernest (fig. 6), âgé de 65 ans, était porteur d'un épithélioma trabéculaire bronchique avec métastases ganglionnaires hilaires et paratrachéales et syndrome de compression de la veine cave supérieure. Il a été soumis à plusieurs cures de moutarde azotée, puis à une radiothérapie médiastinale. A l'époque de l'examen isotopique, l'anémie est discrète (érythrocytes 3,650,000/mm³, Hb 78%, valeur globulaire 1.07), le fer sérique à 90 $\gamma\%$ et le pool plasmatique nettement diminué (2.4 mg), la capacité totale de saturation est de 275 $\gamma\%$. Nous constatons une bonne fixation suivie d'une libération excellente sur la région sacrée; la fixation sternale, en revanche, est fortement inhibée, ce qui est bien compréhensible, cette région ayant été irradiée. La captation hépato-splénique est nettement accrue; sur la région hépatique, on voit apparaître ensuite une baisse de la radioactivité, suivie d'une remontée, certainement imputable à une légère hémolyse. Comme l'incorporation globulaire est presque satisfaisante (80%) et que l'existence d'une érythropoïèse hépatique est exclue dans ce cas, nous sommes enclins à supposer qu'il doit exister un mouvement du fer vers le foie, suivi dans un second temps d'un mouvement partant du foie pour rejoindre la moelle. Les courbes plasmatiques, si elles ne permettent pas les calculs selon Pollycove exposés plus haut, en raison du nombre insuffisant de prélèvements sanguins au cours des 24 premières heures, montrent l'absence de la seconde exponentielle.

Monsieur M. Charles (fig. 6), patient de 59 ans, atteint d'un carcinome bronchique à petites cellules, présentait une hépatomégalie métastatique considérable à croissance rapide. Une cure de moutarde azotée avait été pratiquée sans succès. L'anémie était ici modérée (érythrocytes 3,780,000, Hb 74%, V. G. 0.98), le fer sérique légèrement élevé (186 $\gamma\%$) avec une capacité totale de saturation de 288 $\gamma\%$. L'épreuve isotopique montre une fixation et une libération médullaires modérées mais nettes. De nouveau nous sommes frappés dans ce cas par une fixation dans les réserves fortement augmentée, contrastant avec une incorporation que, par extrapolation, on peut évaluer à 80%. Sur les courbes plasmatiques, nous retrouvons l'absence presque complète de la seconde exponentielle. L'épreuve est interrompue par le décès du malade. Le comptage de fragments prélevés à l'autopsie a permis de confirmer l'existence d'une radioactivité hépatique plus de deux fois supérieure à la radioactivité de la moelle sacrée.

Enfin, le troisième patient, Monsieur K. Gustave (fig. 7), 65 ans, a été traité par hormonothérapie soustractive et additive à plusieurs reprises pour un carcinome prostatique décelé en 1962. Il présente des métastases osseuses et surtout hépatiques. L'anémie modérée (érythrocytes 4,040,000, Hb 73%), légèrement hypochrome (V. G. 0.91), s'accompagne d'une baisse marquée du pool plasmatique (2.2 mg) avec un fer sérique à 84 $\gamma\%$ au début de l'épreuve, à 67 $\gamma\%$ un mois et demi plus tard; la capacité totale de saturation est de 306 $\gamma\%$. L'entrée de l'isotope dans la moelle et sa sortie sont présentes, quoique restreintes; cette fois encore, et d'une manière plus accentuée, le fer gagne précocément les réserves, surtout hépatiques; il paraît les quitter par la suite, pour être finalement incorporé dans les érythrocytes au taux de 80%. En outre, une légère hémo-

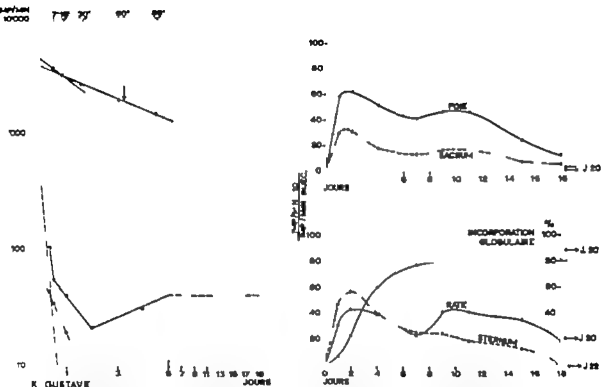


Fig. 7. K. Gustave, carcinome prostatique: Décroissance plasmatique, incorporation globulaire et courbes de radioactivité externe corrigées selon la radioactivité circulante.

lyse est observée dès le neuvième jour La seconde exponentielle paraît presque absente: les calculs effectués selon le modèle de Pollycove, en construisant néanmoins trois exponentielles (puisque l'on sait qu'il existe un mouvement vers les réserves par les courbes de comptages externes) aboutissent à une estimation quantitative des mouvements du fer vers les réserves très légèrement accrus (5.1 mg/j) seulement, au lieu de 18 mg en moyenne dans une hémochromatose (20) Le pool préhéminique est fortement diminué (16 mg).

Nos trois cas d anémie cancéreuse offrent par conséquent ce contraste d une séquestration hépatique nette, d après les courbes de comptages externes, avec l absence de la seconde des trois exponentielles habituelles d une part et une incorporation globulaire presque satisfaisante d autre part. Deux questions se posent alors 1 Pourquoi n observe t-on pas de seconde exponentielle plus importante si les réserves sont accrues? 2 Si le fer va dans les réserves, pourquoi l incorporation n est-elle pas diminuée?

A la première de ces questions, nous pensons pouvoir répondre comme suit Ou bien la «seconde» exponentielle est en fait l exponentielle tardive que nous voyons sur la courbe, confondue éventuellement avec la «troisième» ou bien elle est réellement absente ceci pourrait être dû à une avidité accrue du système réticulo-endothélial pour le fer de sorte que ce métal ne serait pas rendu facilement au plasma comme dans l hémochromatose par exemple. Cependant, pour répondre à la seconde de ces questions, force est d admettre que le fer capté précocément d une manière exagérée par les réserves, revient par la suite lentement au plasma, permettant ainsi à la moelle d effectuer une incorporation presque normale. Cette traversée continuelle des réserves aboutirait toutefois à un appauvrissement progressif du capital utilisable du fer réalisant une sorte de «spoliation interne» et permettrait d expliquer l hyposidérémie classique du cancéreux. En d autres termes, chez le patient atteint de cancer il devrait se développer si sa survie était assez longue, un accumulation de fer dans le foie qui serait alors due non à une résorption accrue primitive du fer (comme dans l'hémochromatose idiopathique) ou à une résorption accrue secondaire à un trouble de l incorporation du fer dans l'hème mais à une avidité tissulaire, en particulier du système réticuloendothélial, d ou l'hyposidérémie qui l opposerait aux deux formes précédentes. Ou peut-être est-ce un trouble du transport du fer qui explique cette déviation de sa voie métabolique normale? Enfin, s agit il vraiment d une liaison au système réticulo-endothélial ou bien faut il incriminer le tissu métastatique lui même, abondant dans les deux der

niers cas? Il n'est pas possible de le dire; cependant les masses néoplasiques ne sont certainement pas seules en cause, si tant est qu'elles le sont, car dans le cas R., qui n'avait aucune métastase dans le foie, la fixation sur cet organe est nettement augmentée, bien qu'à vrai dire moins fortement que chez les autres.

La notion d'une captation accrue du fer par les tissus, a déjà été décrite par VANNOTTI (22) puis KEIDERLING (11) dans l'anémie infectieuse et cancéreuse. En outre, FREIREICH et coll. (7) ont observé expérimentalement une inhibition du lâchage du fer par le système réticulo-endothélial au cours de l'inflammation. L'interprétation que nous proposons de nos résultats serait donc en accord avec les travaux de ces auteurs.

Comme nous l'avons vu, la seconde exponentielle pourrait être invisible parce que très lente et confondue ainsi avec la troisième. Ceci traduirait alors un compartiment labile des réserves non plus d'avidité accrue mais de volume considérable. Pratiquement cela ne changerait d'ailleurs pas le schéma général proposé.

Conclusions

Le modèle élaboré par POLLYCOVE ET MORTIMER permet de calculer la quantité d'Hb réellement synthétisée chaque jour et d'obtenir des valeurs qui paraissent en accord avec le contexte hématologique, ce qui n'était pas le cas de l'ancienne notion de renouvellement globulaire. C'est là probablement le principal avantage de cette méthode longue et compliquée. L'étude des mouvements du fer vers les réserves, par contre, est beaucoup plus sujette à caution: d'une part il est souvent bien difficile, sinon arbitraire, de décider s'il existe deux ou trois exponentielles dans la courbe expérimentale de décroissance plasmatique prolongée; ceci n'est d'ailleurs pas d'une grande importance mais plus ennuyeux est le problème de savoir si une «seconde» exponentielle absente se confond avec la troisième exponentielle par excès du volume du compartiment labile des réserves ou au contraire avec la première par absence de ce compartiment. Une autre difficulté dans le dépouillement de ces résultats réside dans la complexité que jette sur les courbes plasmatiques l'existence si fréquente d'une légère hémolyse. C'est la raison pour laquelle nous n'avons pas insisté sur le calcul du fer «échangeable» (X_{ms} de POLLYCOVE) qui était ainsi impossible à calculer dans la majorité de nos cas. Malgré ces défaillances de la méthode, il nous paraît intéressant de noter ce mouve-

vement vers les réserves beaucoup plus important dans l'hémochromatose secondaire que dans l'hémochromatose primitive.

Résumé

Les auteurs rappellent les arguments qui ont conduit à substituer aux modèles simples des mouvements du fer des modèles plus complexes faisant place à des pools labiles intermédiaires entre le plasma et l'hémoglobine d'une part, les réserves d'autre part. L'application des conceptions de POLLYCOVE chez quelques patients montre que cette méthode permet de calculer la synthèse d'hémoglobine d'une manière très satisfaisante. Les procédés d'interprétation tendant à explorer l'importance quantitative des réserves donnent des résultats plus discutables. Il apparaît cependant que le mouvement du fer vers les réserves est beaucoup plus important dans l'hémochromatose secondaire à l'anémie sidéroachrestique que dans l'hémochromatose primitive. Enfin, dans l'anémie cancéreuse l'analyse des courbes semble montrer une déviation du fer vers les tissus, dont le mécanisme probable est discuté.

Summary

The authors indicate the reasons which have led to the earlier simple concepts of iron metabolism being replaced by more complex ideas, incorporating intermediary labile pools between the plasma and haemoglobin on the one hand ant the stores on the other Applying POLLYCOVE theories in few patients has shown that haemoglobin synthesis can be calculated most satisfactorily by this method. Interpretations based on the quantitative aspects of iron stores give more questionable results. It appears, however that storage of iron is much more considerable in haemochromatosis secondary to iron-resistant anaemia than in primary haemochromatosis. In neoplastic anaemia, the results seem to show that iron is diverted to the tissues the probable mechanism is discussed.

Zusammenfassung

Es wird auf die Gründe hingewiesen, weshalb für die Beurteilung des Eisenstoffwechsels die einfacheren Modelle durch kompliziertere Verfahren ersetzt werden, die auch die labilen intermediären Pools zwischen Plasma und Hämoglobin einerseits und den Reserven andererseits berücksichtigen. Die Anwendung der Auffassung von POLLYCOVE bei einigen Patienten erlaubte, die Hämoglobinsynthese auf eine befriedigende Weise zu berechnen. Jedoch ergeben die Verfahren zur quantitativen Erfassung der Reserven zweifelhafte Ergebnisse. Immerhin scheint die Eisenverschiebung in die Depots bei der sekundären Hämochromatose im Gefolge einer sideroachrestischen Anämie wesentlich stärker zu sein als bei primärer Hämochromatose. Bei Karzinomanämie fand sich eine Abwanderung des Eisens in die Gewebe deren möglicher Mechanismus besprochen wird.

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A Megakaryocyte Stimulating Substance in Normal Human Plasma*

By Bernhard Steinberg and Frank H. F. Cheng

In a previous report it was indicated that fractions associated with normal human serum albumin were found to exert varying effects on the composition of bone marrow and frequently on the cells in the peripheral blood of rabbits (1). Since an heterologous serum and multiple intravenous injections were used, toxicity and an antigen antibody reaction were two of the several problems which required evaluation. The existence of toxicity of human serum for rabbits has been recognized and was demonstrated in our experiments by death of animals within several hours following intravenous injection of 2.2 ml per pound of body weight of the rabbit (table I). This toxic effect was removed after 24 to 48 hours of dialysis of serum (table II) (2). Judd and Steinberg (3) evaluated the effect of a possible immunologic process in vivo and on blood cells in vitro. They found that antigenicity of serum fractions responsible for marrow and blood cell changes were unrelated to biologic activity.

In the case of serum toxicity the response of the animal was rapid and distinctive. As Hueper (4) pointed out, the 'macromolecular hematologic syndrome' such as leukopenia was always transient, lasted for a few hours and was followed by leukocytosis. In our experience the changes in the rabbit due to injection of human serum were characterized by hemolysis and anemia with or without thrombocytopenia. These effects occurred within hours after injection and either disappeared in 24 to 48 hours or caused death of the animals. Fractions associated with normal human albumin produced entirely dissimilar responses, apparently unrelated to toxicity (1, 2).

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The purpose of this presentation is to offer experimental evi dence of presence of a component in normal human serum which is associated with an electrophoretically fast moving part of albumin. This component stimulated an increase in the number of mega caryocytes in bone marrow of rabbits. Studies of the peripheral blood indicated that the stimulation of megakaryocytes was not associated with a concomitant increase of platelets.

Table I

Effect of intravenous injections of normal human serum into rabbits in doses of 2.2 ml per lb of body weight.

No. of animals	No. of injections	No. of dead animals
107	1	92
6	2	6
37	3	37

Death occurred in 2 to 5 hours.

The large number of animals in this series of experiments is due to previous study of changes incident to injections of human serum into rabbits.

Table II

Effect of dialysis of normal human serum on survival of rabbits. The animals were given intravenous injections in doses of 2.2 ml per lb of body weight.

No. of animals	Time of dialysis in hours	No. of dead animals
10	[illegible]	2
8	48	0
5	72	0
15	96	0

Dialysis was done at 5 °C against running distilled water

Methods and Materials

Normal human blood was obtained from blood banks and was collected in ACD anticoagulant solution. The age of the blood varied from 2 to 60 days. The plasma was siphoned off after centrifugation. Several batches of plasma were pooled to make one liter The plasma was dialyzed against running cold tap water for 48 to 72 hours. T one part of the resulting serum were added 3 parts of distilled water and enough sodium tetrametaphosphate to make 0.5% concentration. The chemical composition of the metaphosphate was standardized and prepared to obtain satisfactory purity Prior to the addition of serum, the fluid was adjusted to pH of 4.4 with 4N acetic at tempera-

Dr Edward J Griffith of Monsanto Company generously prepared and standardized the sodium tetrametaphosphate.

ture of 22 °C. and sodium chloride was added to give a 0.043 molar solution. Since differential protein precipitation occurred within a narrow range of pH, sensitive recording instrument was used. The water solution was added to the serum slowly with magnetic stirrers over a period of 30 minutes and the mixture was allowed to stand for 60 minutes at 22 to 27 °C. The resulting precipitate was removed by centrifugation at 2000 rpm for 12 minutes. The precipitate was washed with 3 volumes of 0.1 M sodium acetate t pH of 4 4 The precipitate was dissolved in 0.85% salt solution with volume of saline one fourth of the serum at start of fractionation. The pH of the solution was adjusted to 7.2 with 1 N sodium carbonate and dialyzed against 40 volumes of 0.85% salt solution for 48 hours at 4 to 6 °C. with one change of saline in 24 hours. The solution was filtered through a #14 diatomaceous filter distributed in vials of 30 ml volume, frozen and lyophilized in vacuo. The fraction was labeled C-1

The total nitrogen determinations of C-1 fraction varied from 0.35 to 0.88. Lower or higher values indicated unsatisfactory separation and resulted in negative biological tests. The albumin component was in concentration of 0.017 to 0.039 mg nitrogen per ml of serum. Deviation from this range of concentration failed to show bone marrow activity (3) The globulin components in the fraction, as will be indicated later were not involved in the stimulation of megakaryocytes.

The globulins were separated from the albumin by continuous flow electrophoresis using a barbitol buffer of pH 8.6, ionic strength of 0.02, temperature within the cell of 3 to 5 °C. An electrical field of 40 milliamperes and 600 to 640 volts was established through the filter paper curtains. Paper starch-gel electrophoresis and nitrogen determinations were done on fractions to establish the degree of separation.

After the albumin component was separated from the globulins, the albumin was subjected to further continuous flow electrophoresis under the same conditions as previously The albumin was divided arbitrarily into 3 parts determined by the degree of mobility and labeled slow moving, of moderate mobility and rapidly moving Ultraviolet absorption spectra were determined on albumin of C-1 fraction and compared with crystalline human albumin and fractions other than C-1 obtained with sodium tetrametaphosphate at pH values other than 4 4.

The animals were New Zealand rabbits of either sex and approximately 4 months of age. The fractions were injected intravenously into an ear vein in doses equivalent to 4.4 ml of serum at start of fractionation. The use of the original serum in calculating a unit was found satisfactory for the conditions of the experiment. Prior to the injections, studies were done on the peripheral blood twice day for 3 days. The studies included counts of total leukocytes, erythrocytes, reticulocytes and thrombocytes determination of hemoglobin and hematocrit and differential counts of stained blood preparations. Following the injections of fractions, similar studies were done twice daily for the duration of the injections. The injections were administered either on two successive days with three injections daily in doses of 4 4 ml/l body weight or on alternate days for 10 to 14 times.

As controls for the megakaryocyte content in bone marrow 15 normal rabbits varying in age from 2 to 8 months were killed and the bone marrow from the long bones was fixed in Bouin and formaldehyde, sectioned and stained with hematoxylin-eosin dyes. Megakaryocytes were counted in 100 fields, using 16 mm lens. On the basis of subsequent findings, three criteria for evaluating stimulation of megakaryocytes were established. The criteria consisted in counting the total number of megakaryocytes and megakaryoblasts in 100 fields and determining the presence and the number of foci of these cells grouped close together (table III). After the injections were completed, the animals were killed five days after the last injection and the bone marrow was removed, sectioned, stained, and evaluated on the basis of the three criteria. A total of 85 rabbits, exclusive of the controls, were used in the various phases of the study The number of animals in each phase will be indicated in description of results. Several fractions of

normal human serum other than C-1 but with varying quantities of protein were used as controls and injected into 36 animals under the same conditions as fraction C-1 to determine if other serum protein fractions would produce stimulation of megakaryocytes.

Table III

Criteria for establishing presence of stimulation of megakaryocytes in bone marrow

1. Total number of megakaryocytes were counted in 100 fields using 16 mm lens. All areas in the marrow were counted irrespective of degree of cellularity. The normal range of megakaryocytes varied from 180 to 480 with 2 month old rabbits showing numbers above 250.
2. Differential number of megakaryocytes and megakaryoblasts were determined in 100 fields using 16 mm lens. Normal animals showed presence of 10 to 75 megakaryoblasts per 100 fields.
3. Presence of and the number of foci of megakaryocytes and megakaryoblasts were recorded. The foci were composed of several cells in close proximity to each other. In normal animals, such foci were extremely rare.

Results

Bone marrow of normal rabbits varying in age from 2 to 8 months evaluated in terms of the three criteria for stimulation of megakaryocytes showed presence of 180 to 480 megakaryocytes and 10 to 75 megakaryoblasts in 100 fields with the larger number of cells in the younger animals of two months of age (fig 1). There was no significant crowding of megakaryocytes (fig 2a). During the various periods of study 36 rabbits injected 3 times a day for 2 days or every other day for 10 injections with several different fractions other than C-1 did not show significant increases in megakaryocytes or blasts or crowding of cells.

Fraction C-1 obtained from a single batch of serum and injected into 4 rabbits showed a significant increase of megakaryocytes and blasts, the former increasing from 680 to 740 and the latter from 110 to 160 in 100 fields (fig 1). There was a significant number of foci of hyperplasia of megakaryocytes (fig 2b). Of 4 animals, two were given 3 daily injections for 2 days and the other two were injected on alternate days for 9 and 10 times respectively. No significant changes in the two groups of animals were noted. It would appear that 6 injections in 2 days were effective. Of 49 animals injected with fraction C-1 either 3 times daily for 2 days or every other day for 10 to 14 times, all of them had a fairly consistent although variable stimulation of the number of megakaryocytes, megakaryoblasts and a significant increase of foci of hyperplastic cells. Injections of whole albumin or fractions

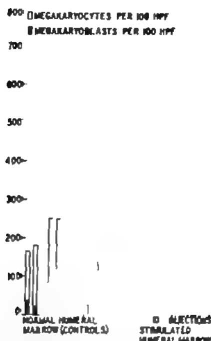


Fig. 1. The left bargram represents a study of the bone marrow of long bones (humeral marrow gives typical representation) of 11 normal rabbits varying in age from 2 to 8 months. The megakaryocytes and blasts were counted in 100 fields using 16 mm objective. The 100 fields (HPF) included areas of marrow of varying degrees of cellularity. The larger number of megakaryocytes were obtained in the 2 month old animals. The right bargram is study of the marrow of 4 rabbits, 4 months of age, injected with C-1 fraction from the same batch of normal human serum obtained by fractionation at pH 4.4 with sodium tetrametaphosphate. The number of megakaryocytes and blasts counted in 100 fields (HPF) shows significant increase as compared to normal rabbits. The 2 bars labeled '6' are of animals injected 6 times over period of 2 days. The bars labeled '9 and 10' are of animals injected 9 and 10 times on alternate days.

of normal human serum other than C-1 did not show an increase of megakaryocytes (table IV).

Various components obtained from fraction C-1 partitioned by continuous flow electrophoresis into globulins and albumin with the latter further divided into 3 parts according to mobility were injected into 15 rabbits. The greatest degree of hyperplasia of megakaryocytes was achieved with albumin with greatest mobility (fig. 3). Studies of peripheral blood of the rabbits injected with fraction C-1 or its albumin component did not show an increase of thrombocytes.

One criterion of satisfactory partition of fraction C-1 was the range of albumin content of 0.017 to 0.039 mg of nitrogen per ml

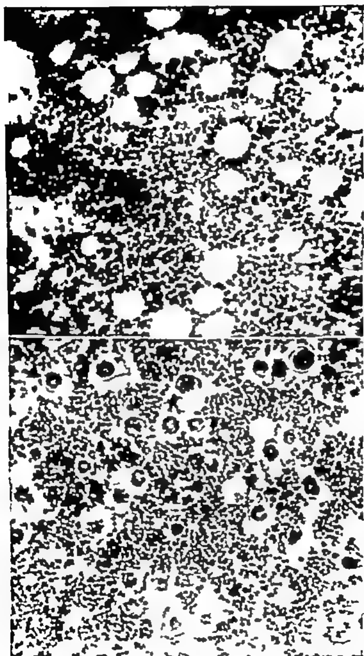


Fig. 1) An Area of humeral marrow of normal 4 month old rabbit. b) Area from the humeral marrow of rabbit given 6 injections of fraction C-1 of normal human serum over period of 2 days and killed 3 days after the last injection. There is significant hyperplasia of megakaryocytes with foci of cells in close proximity to each other
250

Table IV

Effects of intravenous injections into rabbits of fraction (C-1) obtained from normal human serum upon stimulation of megakaryocyte production in the bone marrow. Comparisons with injections of fractions other than C-1 upon stimulation of megakaryocytes.

Fraction injected	No. of animals injected	Effect on the bone marrow of rabbits: Range in 100 fields of the following: No. of megakaryocytes	No. of megakaryoblasts	No. of foci of megakaryocytes
C-1	49	490 to 1250	57 to 285	6 to 31
Total albumin	5	210 to 320	25 to 75	1 to 4
Albumin fractions	12	185 to 340	18 to 62	2 to 8
Globulin fractions (which include some albumin)	19	180 to 350	10 to 42	0 to 4

Interpretation. Fraction C-1 obtained from normal human serum by fractionation at pH of 4.4 with sodium tetrametaphosphate is capable of stimulating hyperplasia of megakaryocytes in the bone marrow of rabbits.

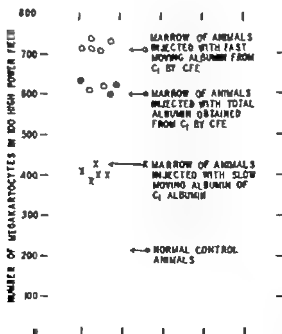


Fig. 3. Fraction C-1 was partitioned by continuous flow electrophoresis into components containing mostly albumin and globulins with the albumin further fractionated by continuous flow electrophoresis into slow and fast moving portions. The fast moving part of albumin showed presence of maximum quantity of substance which stimulated the hyperplasia of megakaryocytes.

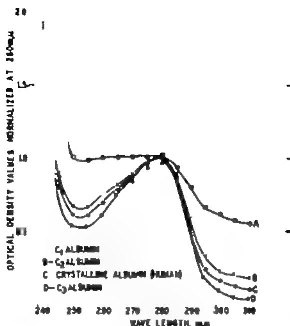


Fig 4 Ultraviolet absorption spectra) Albumin partitioned from fraction C-1 (optical density of one) b),), and d) Human crystalline albumin and albumin partitioned from other fractions (optical density of 0.70 to 0.74).

of serum. Another criterion was the ultraviolet absorption spectrum. A ratio between the optical density of 260 mμ and that of 280 (CD 260/D 280) of nearly one characterized fraction C-1 The optical densities of controls were less than one. Crystalline albumin was 0 70, albumin obtained from other fractions of normal human serum was 0 70 to 0 74 (fig 4)

Discussion

Separation of plasma proteins represents a formidable task made more so if denaturation is to be avoided. The few available procedures including cold-ethanol fractionation were tried but the fractions were either denatured as indicated by inability to effect biological changes and alteration of electrophoretic patterns or the separation was inadequate and volumes were insufficient for pur poses of these studies. Rane and Newhouser (5) suggested that sodium tetrametaphosphate did not denature plasma proteins and at the same time affected a satisfactory separation of proteins. The

chemical was first prepared by WARSCHAUER (6) and subsequent investigations indicated that the method of preparation of the chemical determined the character of fractionation (7) The method of RANE AND NEWHOUSER with certain modifications, adopted in our studies, was found effective in separating protein components of plasma and serum which produced changes in bone marrow and frequently on circulatory blood cells (8) Fractionation with sodium tetrametaphosphate was by no means complete. It furnished only the initial step Further separation by continuous flow electrophoresis indicated that the part of albumin with greater electrophoretic mobility was associated with a hyperplasia of megakaryocytes without a coincident increase of thrombocytes in the peripheral circulation That serum albumin has a transport function because of its ion binding ability and slight permeability of the capillary membrane has been demonstrated (9) If the stimulating effect on megakaryocytes as indicated in the experiments represents a true biological effect by a circulating regulator it may well be assumed that a fraction of albumin serves as a carrier

The use of heterologous serum has raised justifiable doubts of biological significance of stimulation of megakaryocytes. Several observations and experiments described in these and previous studies did not find the heterologous serum to be a factor and furnished evidence in support of the concept of the presence of a true regulator Injections of serum fractions other than C-1 of greater protein content failed to produce stimulation of megakaryocytes. Since albumin is an indifferent antigen in evoking antibodies and sera with high antibody titres were not biologically active (3) the possibility of some antigen-antibody reaction responsible for the stimulating effect of megakaryocytes appeared remote Criteria that characterized true stimulation of megakaryocytes posed another problem. The variation in number of cells in normal animals and difficulties inherent in counting cells on a single plane of an organ were obvious experimental hazards. The following observations aided in establishing criteria Since the bone marrow of a rabbit is a solid tube, multiple sections across the long diameter allowed counts of megakaryocytes which were found reproducible. The increase of megakaryoblasts and foci of closely placed cells served as additional criteria. A study of marrow of normal rabbits of different ages indicated that the younger animals had a greater number of megakaryocytes. The period of 5 days between the last injection of

C-1 fraction and death of animals was selected because of the presumptive 25 day life cycle of a megakaryocyte including a 7 day period of maturation (10).

On the basis of these observations and experiments, the stimulation of megakaryocytes in bone marrow of rabbits by multiple intravenous injections of a fraction of normal human serum may be considered a true hyperplasia. The association of hyperplasia with an electrophoretically highly mobile albumin component suggests that the albumin may serve as a carrier of the stimulant.

Summary

A fraction obtained from normal human serum by separation with sodium tetrametaphosphate at pH of 4.4, 23 °C at specific ionic strength was found to be composed of approximately 10% of total albumin and some globulins. Upon removal of the globulins and division of albumin by continuous flow electrophoresis, the part of albumin with greatest mobility was associated with hyperplasia of megakaryocytes in bone marrow of rabbits, which was not followed by an increase of thrombocytes. Some of the factors which question the interpretation of the stimulation as significant biological phenomenon are discussed.

Résumé

L'étude d'une fraction de sérum humain normal isolée à l'aide de tétramétaphosphate de sodium à un pH de 4,4, à une température de 23 °C et à une concentration ionique spécifique, démontra qu'elle contenait approximativement 10% des albumines totales et quelques globulines. Après l'élimination des globulines et la division des albumines par l'électrophorèse, la fraction d'albumine ayant la plus grande mobilité, provoqua un hyperplasie des mégacaryocytes de la moëlle osseuse chez le lapin, hyperplasie qui ne fut pas suivie d'une augmentation des thrombocytes.

Quelques uns des facteurs qui mettent en question l'interprétation de cette stimulation en tant que problème biologique significatif sont discutés.

Zusammenfassung

Aus normalem menschlichem Serum wurde mit Natriumtetrametaphosphat bei pH 4,4, 23 °C und spezifischem Ionengehalt eine Fraktion gewonnen, die rund 10% der Gesamtalbumine und einige Globuline enthielt. Nach Entfernung der Globuline und Aufteilung der Albumine durch kontinuierliche Flüssigkeitselektrophorese rief die Albuminfraktion mit der grössten Beweglichkeit eine Hyperplasie der Megakaryocyten im Knochenmark von Kaninchen hervor, die nicht von einem Thrombocytenanstieg gefolgt war. Einige Faktoren, die die Interpretation dieser Stimulation als signifikantes biologisches Phänomen in Frage stellen, werden besprochen.

References

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Morphogénèse des cellules erythropoïétiques à deux noyaux par l'action des rayons X

Par E. G. Rondanelli, A. Trenta, E. Magliulo
V. Vannini et G. Gerna

La formation des cellules géantes binucléées, un problème qui a été affronté par divers auteurs sur des matériaux divers et par des techniques différentes depuis le début de notre siècle, a été attribuée à l'un des trois mechanismes suivants: 1. amitose, 2. fusion de deux cellules et, 3. arrêt mitotique. L'amitose a été considérée comme méchanisme fondamental de formation des cellules binucléées, pas seulement dans les organismes inférieurs (30, 20, 33), mais aussi dans les organismes supérieurs (27, 28, 26, 24, 2, 19, 42, 43, 9, 29, 35, 34, 7, 6).

Depuis les premiers rapports de Lewis et Webster (24), de Jordan (19) et de Lewis (23) et aussi plus récemment la formation de cellules binucléées était attribuée à la fusion du cytoplasme de deux cellules accôtées (1, 18, 45, 32, 8, 36). Le méchanisme de l'arrêt mitotique, et plus particulièrement de l'inhibition de la cytodiérèse a été jusqu'ici le plus souvent appelé en cause (21, 44, 14, 22, 5, 15, 3, 46, 4, 37). Cependant il faut reconnaître que la plupart des observations ci mentionnées avaient été conduites sur des préparations fixées et colorées, ce qui pouvait dans quelques cas conduire à des fautes d'interprétation, surtout pour ce qui concerne la direction des phénomènes observés.

Dans le présent travail nous avons investigué les méchanismes morphogénétiques des cellules binucléées dans des cultures «in vitro» de cellules hémopoïétiques irradiées avec des petites doses de rayons X et étudiées par la microphotographie et la microcinématographie en contraste de phase.

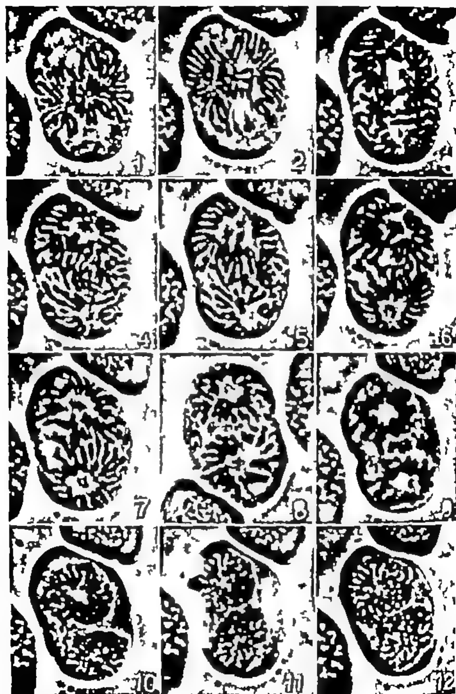


Fig 1 Formation d'une cellule binucléée monucléaire par inhibition de la cytodiérèse
1 Plaque équatoriale vue de côte vec ses chromosomes divisés longitudinalement.
2 7 Migration polaires des chromosomes fils. 8 et 9 Conglutination ana-télophasi-

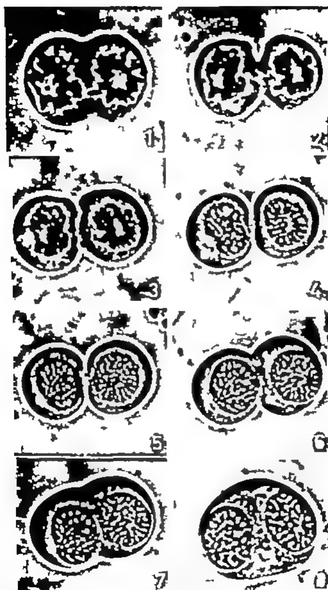


Fig. 2. Formation d'une cellule binucléée mononucléaire par refusion post-mitotique des deux cellules filles. 1–4 Cytodiérèse. 5–8 Réfusion des deux cellules filles probablement par la persistance de très minces filaments cytoplasmiques.

que des chromosomes le sillon équatorial de division cytoplasmique ne se forme pas (on en voit seulement une ébauche dans le N° 9) 10–12 Reconstruction de deux noyaux interphasiques à dimensions égales contenus dans la même cellule

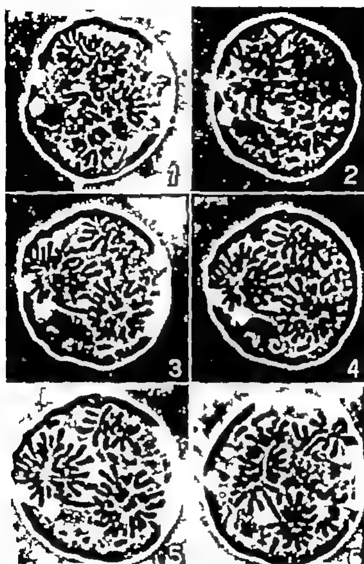


Fig. 3. Arrêt de la mitose en métaphase poly-centrique. Les chromosomes vont se ranger en plusieures étoiles. Les chromosomes sont évidemment divisés longitudinalement et dans les étoiles on observe des chromosomes fils qui se sont séparés complètement. D'autres chromosomes gisent au déhors des étoiles. On observe un pont chromosomique entre les deux étoiles en bas.

Matériel et Méthodes

Le matériel employé était constitué par les erythroblastes basophiles [redacted] polychromatophales provenant de cultures en goutte pendante de rate du triton «Molge Vulgaris L.» Les charactéristiques cytologiques de ce matériel, la technique de culture, ainsi que les modalités de l'irradiation, l'observation en contraste de phase et les prises photo-

graphiques et cinématographiques ont été traitées plus amplement dans d autres publications (38) Les cultures du présent expériment avaient été irradiées avec des petites doses de rayons X (12 – 25)

Résultats

Morphogénèse des cellules binucléées isonucléaires

Nous avons observé les suivantes modalités de formation des cellules binucléées isonucléaires

a) *Arrêt de la mitose en ana-télophase* 1 Inhibition de la cytodiérèse après un déroulement normal de la mitose jusqu à la fin de l anaphase. Le sillon équatorial de division cytoplasmique ne se forme pas, ou, s'il se forme, ne croît pas vers la profondeur et la cellule va reconstruire deux noyaux intercinétiques à partir des chromosomes des deux étoiles filles anaphasiques. Chaque noyau contient évidemment un nombre dyploide de chromosomes et les deux noyaux ont des dimensions parfaitement égales.

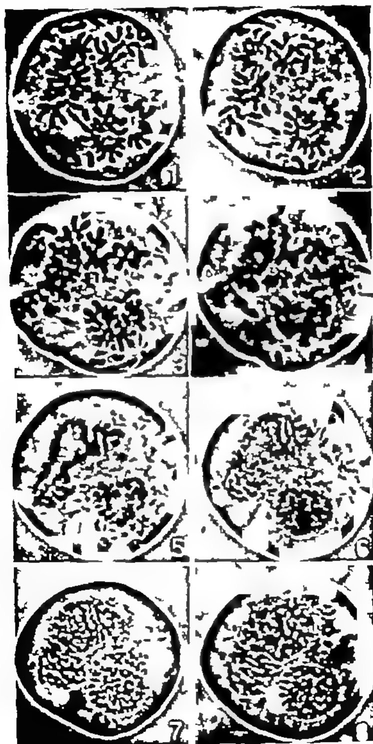
b) *Réfusion post-télophasique de deux cellules filles originées d'une cytodiérèse normale* (fig 2) Les deux cellules filles se séparent complètement, mais restent intimement accôtées et successivement fondent leur cytoplasmes dans un seul originant ainsi une cellule binucléée.

Morphogénèse des cellules binucléées anisonucléaires

Ces cellules binucléées qui, comme le dit le nom possèdent noyaux aux dimensions inégales, s originent selon l une des suivantes modalités

a) *Arrêt de la mitose en métaphase bi-polycentrique* (fig 3 et 4) Si la métaphase bloquée est à deux groupements chromosomiques caractérisés par un différent nombre de chromosomes, ceux-ci vont constituer directement deux noyaux intercinétiques qui restent compris dans la même cellule. Si la métaphase bloquée est à plus de deux groupements chromosomiques, la reconstruction de deux noyaux fils se produit à partir de la préalable conglutination des divers groupements chromosomiques dans deux seules masses de matériel chromatinien, contenues dans la même cellule.

b) *Arrêt de la mitose en anaphase asymétrique* (fig 5 et 6) Le différent nombre de chromosomes qui ont migré vers les deux pôles et la successive inhibition du clivage cytoplasmique conditionnent la formation d une cellule binucléée anisonucléaire.


1
2
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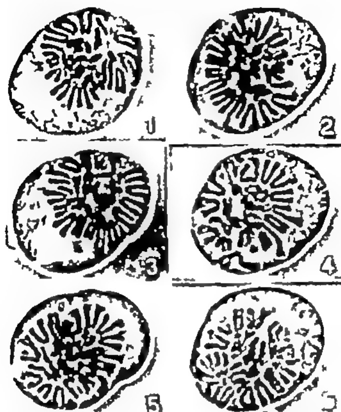


Fig 5. Métaphase dans une cellule d'où originera une cellule binucléée anisonucléaire On observe les mouvements de la plaque équatoriale à l'intérieur de la cellule et la division longitudinale des chromosomes.

c) *Arrêt de la mitose en ana-télophase tri-pluripolaire* A la conglu tination des étoiles chromosomiques en deux seules masses chromatiniennes, suit l'inhibition du clivage cytoplasmique, avec ou sans for mation d une ébauche de sillon équatorial (les No. 3 4 et successives de la fig 6 pourraient constituer un exemple aussi de cette modalité) Pour l inégale distribution des chromosomes dans les deux noyaux, il en résulte une cellule binucléée anisonucléaire

Fig 4 Même mitose de la fig. 3. Formation d une cellule binucléées anisonucléaire par refusion des chromosomes d'une métaphase ploycentrique en deux seules masses chromatiniques (N° 4) d'ou ont se reconstruire deux noyaux interphasiques à differentes dimensions. D'un chromosome aberrant, que l on peut voir en bas dans la cellule dans la N° 4 origine un micro-noyau supplémentaire (5–8)

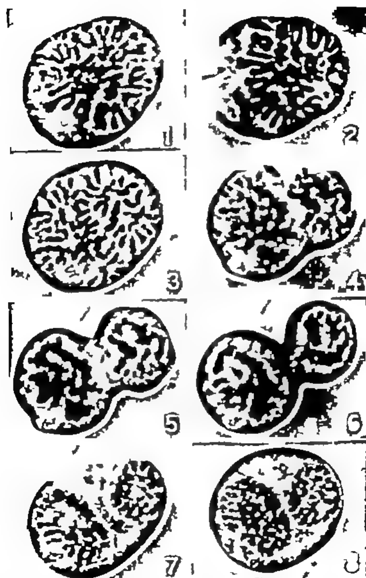


Fig 6 Même cellule de la fig 5 d'où, par anaphase asymétrique et inhibition de la cytodiérèse, va se former une cellule binucléée anisonucléaire. 1 3 Migration très irrégulière des chromosomes fils avec formation de nombreuses petites étoiles groupées dans deux groupements majeurs. 4 et 5 Conglutination des chromosomes en deux masses chromatiniques de diverses dimensions, et formation d'un sillon asymétrique de division cytoplasmique. 7 et 8 Le sillon de division cytoplasmique disparaît [illegible] deux noyaux interphasiques de dimensions inégales vont reconstruire des étoiles asymétriques ana-télophasiques.

Discussion

Il résulte de ce que nous avons ici rapporté que la formation de cellules binucléées isonucléaires après irradiation trouve son origine, excepté le mechanisme de l'arrêt en métaphase bicentrique à étoiles chromosomiques égales (d'autre part assez rare) dans des troubles mitotiques généralement moins graves que ceux qui portent à la formation des cellules binucléées anisonucléaires. Dans ce dernier cas on observe outre à l'inhibition de la cytodiérèse, des altérations des chromosomes comme la «stickiness» et la pycnose, et souvent un plus grave dérangement de la disposition métaphasique et anaphasique conditionnent d'autre part une plus facile conglutination des groupements chromosomiques dans les méta phases tri-pluri-centriques et dans les ana télophases tri-pluri polaires.

Pour ce qui concerne la refusion de deux cellules filles qui s'étaient auparavant complètement détachées, nous ne sommes pas en gré d'en définir les causes bien que celles-ci doivent être recherchées dans des modifications des propriétés mécaniques et physico-chimiques de la membrane cellulaire. Sur la base des observations sur cellules vivantes (37 38) il paraît assez difficile d'accepter une génèse des cellules binucléées par amitose, c'est à-dire par division directe du noyau interphasique. Même si cela pouvait effectivement arriver nous pouvons déclarer que dans nôtre très longue expérience dans le domaine de la microscopie en contraste de phase nous n'avons jamais observé une amitose, ou une division directe d'un noyau interphasique, exception faite pour la segmentation des noyaux des granulocytes mûrs.

Quels peuvent être les mécanismes intimes cellulaires conditionnant l'inhibition de la cytodiérèse? C'est un problème auquel il est assez difficile de repondre, parce que les mécanismes mêmes qui guident la cytodiérèse normale ne sont pas complètement connus. Il a été demontré qu'il n'est pas nécessaire que les chromosomes soient présents pour que le sillon équatoriale se forme (25) même si la masse de cytoplasme des cellules filles parait conditionnée par la masse des chromosomes. Il a été aussi demontré que quelquefois la cytodiérèse peut se produire aussi en présence d'une lésion assez grave du fuseau (10 47) Il en résulterait par élimination que les centres polaires soient les vrais responsables de la division cytoplasmique, ce qui a été soutenu par DAN ET

NAKAJIMA (11) et serait aussi démontré par le fait que dans les mitoses monopolaires d'œuf d'oursin la cytodiérèse ne se produit pas, ou elle se produit après la formation d'un deuxième pôle (31). Selon MAZIA les centres polaires émettraient un «signal» pour la division cytoplasmique. Si la lésion des centres polaires se produit avant que le «signal» ait été émis, la cytodiérèse est inhibée. Si elle se produit après que le «signal» ait été émis, la cytodiérèse n'est pas inhibée. L'émission du «signal» ou la phase critique correspondante serait située dans l'anaphase, comme il est démontré par les expériments de HIRAMOTO (17). Il serait donc admissible que les rayons X agissent à niveau des centres polaires, comme il a d'autre part été démontré par les phénomènes de pluripolarité ou de pluricentrisme que nous avons observé. D'autre part d'autres auteurs (16, 39, 40) ont attribué un rôle aux centres polaires dans l'inhibition mitotique par rayons X. Le fait que le sillon cytoplasmique peut quelquefois se reformer, mais successivement regresser pourrait être expliqué avec un «signal» anormal émis par des centres polaires lésionnés.

Un problème très intéressant est celui de la possibilité que des cellules binucléées entrent en mitose. LEWIS (23) soutenait que les cellules binucléés peuvent encore se diviser. Selon cet auteur si la cellule binucléée avait été originée par une amitose, dans la nouvelle mitose on aurait dû avoir un seul fuseau, tandis que deux fuseaux auraient été observables quand la cellule binucléée avait été originée par une mitose sans cytodiérèse. LEWIS (23), en admettant que tant la mitose que l'amitose peuvent pareillement conduire à la production de cellules binucléées mésenchymales, soutient que la première modalité pourrait être invoquée quand dans la cellule on peut compter seulement deux centrioles, tandis que la présence de quatre centrioles indiquerait que la cellule binucléée se serait originée d'une mitose ne pas suivie par la cytodiérèse.

BEAMS ET KING (3), en étudiant le foie regénérant de rat, avaient soutenu la possibilité que les cellules binucléées puissent entrer en mitose, avec les chromosomes des deux noyaux formant une seule plaque équatoriale à laquelle suivrait l'anaphase et la réconstruction de deux noyaux tetraploïdes contenus dans deux cellules séparées si la cytodiérèse se produit, ou contenus dans la même cellule si se répète l'inhibition de la cytodiérèse. Dans ce dernier cas la répétition du procès entier porterait à la formation de noyaux octoploïdes. Aussi SCHWARZ (41) avait soutenu que les

érythroblastes géants binucléés humains pouvaient entrer en mitose. Celle-ci était un caryocinèse tetrapolaire avec deux fuseaux, qui donnait origine à une cellule binucléée capable à son tour d'entrer à nouveau en mitose. La possibilité que les cellules binucléées puissent entrer en mitose, bien que n'ayant pas été directement affrontée, avait été quand même entrevue aussi par R. Di Guglielmo (13) et G. Di Guglielmo (12). Nous avons suivi dans le temps nos cellules binucléées, mais nous ne les avons jamais observées entrer en mitose. Schwarz a souligné qu'il est essentiel pour que ces cellules entrent en mitose, que la fonction du fuseau soit parfaitement conservée. Peut-être dans notre expériment l'irradiation avait déterminé une inhibition préprophasique dans les cellules binucléées. Mais il faut adjoindre que dans d'autres occasions (par exemple dans les conditions hémopatiques) nous n'avons jamais observé des cellules binucléées entrer en mitose.

Résumé

Dans des cultures «in vitro» de tissus hémopoïétiques l'irradiation par des faibles doses de rayons X peut conduire à la formation de cellules érythropoïétiques binucléées iso- et aniso-nucléaires par l'incapacité du cytoplasme de se diviser en séparant les noyaux fils issus de l'évolution catachromatique des chromosomes de métaphases bi-pluricentriques et ana-télophases bi-pluripolaires. Le nombre des chromosomes dans chaque groupement métaphasique ou ana-télophasique conditionne la grandeur du noyau résultant et la iso- ou l'anisonucléarité des cellules binucléées formées. Des cellules binucléées isonucléaires peuvent se former aussi par la fusion du cytoplasme de deux cellules filles qui auparavant étaient complètement séparées en restant cependant strictement accolées. Les mécanismes intrinsèques de la formation des cellules binucléées sont aussi discutés.

Summary

In irradiated hemopoietic tissues «in vitro» the formation of iso- and aniso-binucleated erythropoietic cells is caused by the failure of cytoplasm to divide and separate daughter nuclei reconstituted by the chromosomes of bi-pluri-centric metaphases and bi-pluri-polar ana-telophases. The size of the two nuclei, and the resulting iso- or anisonuclearity is in relation with the number of chromosomes from which each nucleus was reconstituted. Binucleated cells may also result by fusion of the cytoplasm of two neighbouring cells previously issued from normal mitosis. Intrinsic mechanisms of the formation of binucleated cells are discussed.

Zusammenfassung

In Kulturen von bestrahlten hämopoetischen Geweben entwickeln sich erythropoietische zweikernige iso- und anisonukleäre Zellen, weil sich das Zytoplasma nicht teilen und dabei die aus den Chromosomen von bi- und pluricentrischen Metaphasen und von bi- und pluripolaren Ana-Telophasen wieder aufgebauten Tochterkerne nicht trennen kann. Die Größe jedes Kernes und die folgende Iso- und Anisonuklearität

hängen von der Zahl der Chromosomen ab, aus denen sich jeder neue Kern bildet. Zweikernige Zellen können auch aus der Vereinigung des Zytoplasmas von zwei benachbarten Zellen entstehen, die aus einer soeben beendeten normalen Mitose hervorgegangen sind. Die inneren Mechanismen der Bildung zweikerniger Zellen werden erörtert.

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Thrombozytenstoffwechsel und Freisetzung von 5 Hydroxytryptamin*

Von H. Schievelbein

Nikotin setzt 5-Hydroxytryptamin (Serotonin, 5-HT) aus den Depots des Meerschweinchendarms, des Rattendarms und des Gehirns frei (1). In früheren Untersuchungen stellten wir am Modell der isolierten Kaninchenthrombozyten fest, daß die Freisetzung von 5-HT abhängig ist von der Inkubationsdauer, von der Liberatorkonzentration, von der Thrombozytenkonzentration, nur in sehr geringem Umfang von der Inkubationstemperatur, aber sehr stark vom pH der Inkubationsflüssigkeit (2). Die in Ansätzen mit Thrombozyten zu beobachtende Spontanfreisetzung und die Freisetzung durch Nikotin können durch Hydrazinsulfat und Hemmkörper der Monoaminoxydase, z.B. Isonikotinsäure-isopropylhydrazid (Marsilid, Iproniazid) und 1-[2-(Benzylcarbamoyl)äthyl]-2-isonicotinoylhydrazin (Nialamid) verhindert werden (3).

In Plasma suspendierte Thrombozyten nehmen gegen ein Konzentrationsgefälle 5-HT auf. Diese Speicherung von 5-HT durch Thrombozyten ist durch Nikotin und andere Liberatoren hemmbar. Die Hemmung der 5-HT-Speicherung durch Nikotin kann ihrerseits wieder durch die erwähnten Hydrazinderivate aufgehoben werden (3).

Der Mechanismus der Freisetzung von 5-HT durch Nikotin ist nicht geklärt. Wir vermuteten, daß die Entspeicherung der Thrombozyten eine Folge der durch Nikotin verursachten Erhöhung der Membranpermeabilität der Blutplättchen sein könnte (4). Es kann angenommen werden, daß die 5-HT Konzentration in den Throm-

* Diese Untersuchungen wurden mit Hilfe der Deutschen Forschungsgemeinschaft durchgeführt.

bozyten durch einen aktiven, energieverbrauchenden Transport des Amins in die Zelle aufrechterhalten wird. Normalerweise fällt die «Spontanfreisetzung» gegenüber der aktiven Speicherung nicht ins Gewicht, so daß die 5-HT Konzentration in den Thrombozyten konstant bleibt. Ein Verlust an Transportenergie müßte aber zum Absinken der 5-HT Konzentration in den Plättchen führen und sollte sich durch ein erniedrigtes Niveau an energiereichen Verbindungen, in erster Linie an ATP und an Aktivität der sie liefernden Enzyme zu erkennen geben.

Nach Löhr und Waller (5) ist, unter anderen Enzymen, die Glukose-6-phosphatdehydrogenase (G-6-PDH) ein Prädilektionsort für die im Verlaufe der physiologischen Zellalterung eintretenden Stoffwechselstörungen. Die enge Koppelung dieses Enzyms mit dem Kohlenhydratstoffwechsel und damit mit der Energiegewinnung ließ es als Indikator für unsere Problemstellung als geeignet erscheinen. Wir maßen daher die Aktivität der G-6-PDH in Thrombozyten unter den Bedingungen der 5-HT Freisetzung durch Nikotin. Außerdem bestimmten wir die Aufnahmefähigkeit der Thrombozyten für 5-HT nach Entspeicherung mit Nikotin im Vergleich zu normalen Thrombozyten. Als Repräsentant der energiereichen Verbindungen bestimmten wir ATP das nach Born et al. (6) als Bindungspartner für die Speicherung des 5-HT in Thrombozyten in Betracht kommt.

Methodik

Zu den Ansätzen wurde das durch differenziertes Zentrifugieren gewonnene sogenannte «plättchenreiche» Plasma aus Kaninchenblut verwendet. Es enthielt im Mittel 404.000 Thrombozyten/mm³. Inkubationsdauer 60 Min. bei pH 7,3. 5-HT (Kreatininsulfatkomplex) und Nikotin (freie Base) wurden in isotonischem Phosphatpuffer gelöst den Ansätzen zugefügt. Volumen der Ansätze 3 ml (Entspeicherung) 10 ml (Beladung) und 20 ml (G-6-PDH).

Nach Inkubation wurden die Thrombozyten abzentrifugiert, die Plättchen durch Zugabe von 0,1 n HCl aufgelöst und anschließend enteiweißt. Im Überstehenden wurde 5-HT entweder colorimetrisch oder fluorometrisch nach Udenfriend u. Mitarb. (7) bestimmt.

ATP wurde nach dem von Lamprecht und Trautschold (8) beschriebenen Vorgehen bestimmt.

Die Messung der Aktivität der G-6-PDH wurde mit der «Biochemica-Test Combination Boehringer» für die Bestimmung der Aktivität des Enzyms in Erythrocyten vorgenommen. Die hier verwendete Methode lehnt sich an die von Kornberg und Horecker (9) angegebene an. Die isolierten Thrombozyten wurden zweimal mit NaCl-Lösung gewaschen und wie für Erythrozyten vorgeschrieben, mit Digitonin «hämolysiert». 0,1 ml des «Hämolysates» verwendeten wir zum Ansatz. Die Aktivität ist ausgedrückt in Millieinheiten (IUB*)/8 × 10⁹ Thrombozyten. Nikotin hat in der von

uns verwendeten Konzentration keinen Einfluß auf die Aktivität des Enzyms im Thrombozytenhämolysat.

Nikotin wurde nach Isolierung durch alkalische Wasserdampfdestillation mit einer Methode bestimmt, welche auf der Extinktionszunahme des Alkaloids bei 259 mμ beim Wechsel vom neutralen zu sauren Milieu beruht (Beiträge zur Tabakforschung, Heft 8, S. 315 (1962)).

Die Zentrifugation von Thrombozytenhomogenat erfolgte gegen einen Dichtegradienten in Anlehnung an das von Bonw (10) beschriebene Vorgehen.

Bei den Speicherungsversuchen der Abb. 1 wurde folgendermaßen verfahren: Plättchenreiches Kaninchenplasma wurde auf mehrere Ansätze verteilt. Nach Bestimmung des Ausgangswertes an 5-HT (= 100% in Abb. 1) wurde ein Teil der Ansätze mit Nikotin (0,005 m) und ein anderer ohne Nikotin 60 min lang inkubiert. Nach der Inkubation wurde das Thrombozytensediment zweimal mit NaCl-Lösung gewaschen und anschließend in Kaninchenplasma resuspendiert. Allen Ansätzen wurde 5-HT (600 μg/ml) zugesetzt. Nach Inkubation wurde 5-HT in den Thrombozyten, wie oben beschrieben, bestimmt.

Ergebnisse

1. ATP. In Tabelle I ist der Gehalt an ATP, 5-HT und Nikotin von Kaninchenthrombozyten verzeichnet. Nach Inkubation mit Nikotin nimmt der 5-HT-Gehalt ab, Nikotin wird aufgenommen, der ATP-Gehalt bleibt unverändert.

Tabelle I

Der Gehalt an ATP, 5-HT und Nikotin in Thrombozyten von Ansätzen nach Inkubation ohne und mit Nikotin. Mittelwerte und mittlerer Fehler in μg/Ansatz aus je drei Ansätzen. Nikotinkonzentration 5×10^{-3} m.

Ansatz	ATP	5-HT	Nikotin
ohne Nikotin	111±14	89±5	0
mit Nikotin	109±10	54±7	54±15

Es besteht die Möglichkeit, daß zwar der Gesamtgehalt der Thrombozyten an ATP konstant bleibt, sich die Verteilung von ATP innerhalb der Thrombozyten aber ändert. Wir untersuchten daher die aminenthaltende Granulafraktion der Thrombozyten nach Zentrifugation über einen Dichtegradienten aus Saccharose. Die Ergebnisse, die in Tabelle II verzeichnet sind, zeigen, daß auch in der Granulafraktion bei der Freisetzung von 5-HT die ATP-Konzentration nicht abnimmt, obwohl eine erhebliche Menge 5-HT freigesetzt wurde. Nikotin war in der Granulafraktion nicht mit Sicherheit nachweisbar.

Report of the Commission of Enzymes, Pergamon Press 1961. 1 E ist die Enzymmenge, die bei 25 °C in 1 min 1 μM Substrat pro 1000 ml umsetzt. 1 Millieinheit = 1/1000 E.

Tabelle II

Der Gehalt an ATP und 5-HT in der Granulafraktion von Thrombozyten vor und nach Inkubation mit Nikotin. Mittelwerte und mittlerer Fehler aus je 7 Ansätzen in μg/Ansatz. Nikotinkonzentration 5×10^{-4} m.

Ansatz	ATP	5-HT
ohne Nikotin	58±6	38±9
mit Nikotin	56±4	16±2

Thrombozyten enthalten ATPase (11). Es wurde eine Aktivierung dieses Enzyms durch Reserpin diskutiert, da die Freisetzung von Aminen in Gehirn und Leber mit einem Verlust an ATP verbunden ist. Wir setzten daher unseren Ansätzen p-Chloromercuribenzoat zu. Diese Verbindung hemmt unter anderen Enzymen auch stark die ATPase. Der Inhibitor verursachte keine Änderung des ATP-Gehaltes von Thrombozyten, er führte aber zu einer Freisetzung von 5-HT, die größer war als die durch Nikotin hervorgerufene, dabei addierte sich die Freisetzung von 5-HT durch p-Chloromercuribenzoat und Nikotin.

2. *Glukose-6-phosphatdehydrogenase.* Aus Tabelle III ist ersichtlich, daß die Aktivität des Enzyms innerhalb der Inkubationszeit um ca. 10% und nach Inkubation mit Nikotin um ca. 20% abnimmt. Die Unterschiede in der Aktivität sind statistisch nicht signifikant, doch sind die Werte nach Inkubation niedriger oder mindestens gleich den Ausgangswerten, so daß hieraus die Tendenz zum Absinken der Aktivität abgeleitet werden kann. Die Schwan-

Tabelle III

Aktivität der Glukose-6-phosphatdehydrogenase in Thrombozyten unter dem Einfluß von Nikotin. Aktivität in Millieinheiten (IUB)/8×10^{9} Thrombozyten. Nikotinkonzentration 5×10^{-4} m.

Ansatz N	Aktivität ohne Inkubation	nach Inkubation ohne Nikotin	nach Inkubation mit Nikotin
1	94	80	69
2	348	323	317
3	44	43	29
4	131	132	113
5	127	121	80
6	49	18	18
Mittelwert und mittlerer Fehler	133±55	120±44	102±41

kungen in der Aktivität zwischen den einzelnen Ansätzen ergibt sich aus der Tatsache, daß die Thrombozytenzahl der Ansätze sehr stark schwankt, die Berechnung aber auf Grund des Mittelwertes der Thrombozytenzahl aller Ansätze vorgenommen wurde.

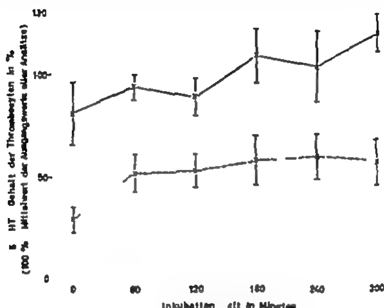


Abb. 1 Aufnahme von 5-HT durch isolierte Kaninchenthrombozyten nach Spontanentspeicherung (———) und nach Entspeicherung durch Nikotin (- - - -). Mittelwerte und mittlere Fehler

3. 5-HT-Speicherung. Sollte die Aktivität der G-6-PDH ein Indikator für einen gestörten Energiehaushalt der Zelle sein, so müßte die Wiederbeladung der Thrombozyten mit 5-HT nach Entspeicherung mit Nikotin vermindert sein. Wir untersuchten daher die Speicherungsfähigkeit der Thrombozyten nach dem Einfluß von Nikotin. Wie aus der Abb. 1 ersichtlich, sind mit Nikotin entspeicherte Thrombozyten nicht in der Lage, die 5-HT Depots wieder bis zu dem Niveau der unbehandelten Thrombozyten aufzufüllen. Der Unterschied der Mittelwerte der Meßwerte bei 300 min wurde berechnet und ist mit einem P von 0,0025 statistisch signifikant.

Diskussion

Die Möglichkeit der Bindung von 5-HT in den Thrombozyten an ATP oder ein anderes Polyphosphat ist häufig diskutiert worden.

Die Beweise, die für diese Bindung bisher vorgelegt wurden, sind indirekter Natur. Eine besondere Stütze dieser Ansicht ist die Feststellung, daß der 5-HT-Gehalt und der ATP-Gehalt in verschiedenen Geweben parallel gehen (10), und daß die Freisetzung von Aminen mit einem Verlust an ATP einhergehen kann. Allerdings ist die Freisetzung von 5-HT aus Thrombozyten durch Reserpin nicht mit einem ATP-Verlust verbunden (10). Die Freisetzung von 5-HT durch Nikotin verändert den ATP-Gehalt der Plättchen nicht, es tritt auch keine Änderung der Verteilung des ATP innerhalb der Plättchen ein, da nach der Inkubation mit Nikotin die Granulafraktion noch den ursprünglichen ATP-Gehalt aufweist. Es besteht also keine Korrelation zwischen dem ATP-Gehalt der Thrombozyten und ihrem Gehalt an 5-HT. Auch andere Autoren kommen zu dieser Auffassung, so ist z. B. der 5-HT-Gehalt der Thrombozyten bei Fällen von Thrombasthenie normal (12, 13, 14), während der Gehalt an ATP in den Thrombozyten bei dieser Erkrankung vermindert sein kann (15).

Nach Entspeicherung mit Nikotin ist die Speicherungsfähigkeit für 5-HT vermindert (Abb. 1). Wir konnten nicht mit Sicherheit entscheiden, ob geringe Reste von Nikotin in den Thrombozyten zurückgeblieben waren, welche die Aufnahme hemmen könnten. Die Verminderung der Speicherfähigkeit und der Verlust der G-6-PDH-Aktivität können zufällig parallel gehen. Löhr und Waller (5) sahen bereits nach Waschen der Thrombozyten einen erheblichen Abfall der Enzymaktivität. Diese Behandlung vermindert aber den Gehalt an 5-HT nicht oder nur in einem sehr geringen Umfang. Die Minderung der Speicherung ist prozentual größer als der Verlust an G-6-PDH-Aktivität, doch kann dies auf einem Verlust an anderen für die Speicherung wichtigen Enzymen beruhen. Es kann daher zum gegenwärtigen Zeitpunkt nicht entschieden werden, ob die Hemmung der Aufnahme von 5-HT durch Nikotin auf der Beeinträchtigung des aktiven Transportsystems zustande kommt, oder ob die Hemmung der Speicherung durch die parallel laufende Freisetzung hervorgerufen wird. Wahrscheinlich sind beide Prozesse im Spiel.

Ich danke Frl. L. Eckert und Frl. G. Schwitzer für gewissenhafte Mitarbeit.

Zusammenfassung

5-Hydroxytryptamin (Serotonin, 5-HT) wird durch Nikotin aus isolierten Kaninchenthrombozyten freigesetzt; dieser Vorgang ist mit einer Aufnahme von Nikotin

durch die Thrombozyten verbunden. Der Gehalt der Thrombozyten und der aminenthaltenden Granulafraktion an ATP ändert sich bei der Freisetzung durch Nikotin nicht. In 5-HT-haltigem Plasma suspendierte Thrombozyten nehmen das Amin gegen ein Konzentrationsgefälle auf. Nach Inkubation mit Nikotin und Resuspension ist die Fähigkeit zur Speicherung vermindert. Unter diesen Bedingungen verlieren die Thrombozyten ca. 20% ihrer Aktivität an Glukose-6-phosphatdehydrogenase.

Summary

Nicotine releases 5-hydroxytryptamine (serotonin, 5-HT) from isolated rabbit blood platelets. This process is connected with uptake of nicotine by platelets. The ATP content of platelets and their amine storage granules is not changed during release of 5-HT by nicotine. Platelets incubated in plasma containing 5-HT take up the amine against concentration gradient, after incubation with nicotine and resuspension, this storage process is diminished. Under those experimental conditions, the activity of glucose-6-phosphate dehydrogenase of the platelets is diminished by 20%.

Résumé

La 5-hydroxytryptamine (Sérotonine 5-HT) est libérée des thrombocytes isolés du lapin par la nicotine cette libération est liée à une résorption de nicotine par les thrombocytes. Le contenu des thrombocytes et de la fraction des granulations contenant de l'ATP ne change pas, lors de la libération par la nicotine. Les thrombocytes en suspension dans un plasma contenant de la 5-HT résorbent l'amine malgré la différence de concentration.

Après incubation avec de la nicotine et résuspension, la capacité de résorption diminue. Dans ces conditions les thrombocytes perdent à peu près 20% de leur activité en glucose-6-phosphatdehydrogenase.

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Life Span and Sites of Sequestration of Normal Erythrocytes in Normal and Splenectomized Mice and Rats*

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Studies of hereditary and acquired red cell abnormalities in man and of experimentally altered erythrocytes in man and laboratory animals suggest that quantitative rather than qualitative factors determine the sites of red cell destruction (1—6). Thus, erythrocytes which are only slightly abnormal or mildly injured pass through the liver and are sequestered mainly by the spleen. With increasing injury, hepatic sequestration becomes more prominent and with severe injury this type of red cell removal predominates. On the basis of these findings, it has been postulated that the reticuloendothelial system (RES), particularly the liver and spleen, plays a major role in the destruction of physiologically aged erythrocytes. Experiments in laboratory animals designed to test this hypothesis have resulted in conflicting evidence depending, in part, on the species studied and, in part, on techniques employed. MIESCHER (7) and VON EHRENSTEIN AND LOCKNER (8) have reported that in rabbits the bone marrow is the major RES site responsible for destruction of normal erythrocytes. Studying rats, HALL et al. (9) and TIZIANELLO et al. (10) found a decrease, whereas THOMPSON et al. (11) and BELCHER AND HARRISS (12) found a prolongation of the red cell life span following splenectomy, permitting no conclusion regarding the role of the spleen in the species.

The present experiments were designed to study the red cell life span in in bred normal or splenectomized mice and rats

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employing radioactive sodium chromate^{51} of high specific activity When the circulating labeled erythrocytes had reached 10 / of initial values, the animals were sacrificed and the radioactivity of all major RES sites, including bone marrow was determined.

Methods

DBA/2 brown male and female mice (weighing 22—29 [illegible] and white Sprague-Dawley male rats (weighing 350—450 [illegible] were housed one animal per cage and fed with Rockland laboratory animal diet and water ad libitum. All animals were kept in ordinary cages on wood shavings.

Splenectomy was performed under ether anesthesia t least four weeks before the beginning of the experiment. Isologous, heparinized blood was taken from pentothal-anesthetized mice and rats from the inferior vena cava and was labeled with 20 to 30 μc/ml sodium chromate^{51} (Chromitope sodium, E. R. Squibb & Sons, New York, specific activity 218—369 mc/mg Cr^{51}). The bloods were incubated at room temperature for 30 minutes after which the incubation was terminated by addition of 8—10 mg/ml sodium ascorbate. The erythrocytes were washed twice with saline, and packed red cells were brought to the original volume with normal saline. 0.25 ml and 0.5 ml of the labeled blood were injected into the tail vein of each of 43 mice and 42 rats, respectively The animals were bled at regular time intervals beginning t 24 hours following the administration of the labeled blood. 0.02 ml blood was taken from the cut tail end into 4 ml 0.1% sodium carbonate. The hemoglobin concentration of the blood sample was determined photometrically at 540 mμ, employing Leitz photometer Radioactivity was determined in multiscaler well counter (background 130—150 cpm). Sufficient counts were obtained to reduce errors to ±2%

The total radioactivity of the injected erythrocytes was calculated from an 0.1 ml aliquot of labeled blood. The radioactivity of the blood determined at 24 hours was taken as 100%, and the radioactivity of the samples on the following days was expressed as per cent of the 24-hour sample counted on the particular day The data were not corrected for elution.

The animals were sacrificed by dislocation of the cervical vertebrae when the erythrocyte life span reached the 10% level. The spleen, liver kidneys, lungs, and heart were removed, weighed, and radioactivity of these samples was determined in the well-type scintillation counter Total organ radioactivity was calculated by multiplying specific activity (cpm/g) by the weight of the organ. Muscles and connective tissue were stripped from the bones (scapulae, humeri, radii, ulnae vertebrae, sacrum, pelves, femora, tibiae, and fibulae) and "bone marrow" radioactivity was determined by counting all the bone fragments obtained. Specific activity was not calculated for "bone marrow" as the weight of marrow could not be determined. Organ radioactivity was then ex pressed as per cent of the total injected activity Standard deviations were calculated according to Snedecor (13)

Results

Mice The chromium^{51} half-life of the erythrocytes (T/2 Cr^{51} Rbc) was 20.2±3.3 (S D) days for the normal and 20 2±2 8 days for the splenectomized mice. The mean erythrocyte survival approximated from the life span curves was 63 days in normal and 67 days in splenectomized mice (fig 1)

Table I

Mean specific Cr^{51} activity in organs

	Spleen		Liver		Kidneys		Lungs		He
	Normal**	Splx***	Normal	Splx	Normal	Splx	Normal	Splx	Normal
Mice	53,300	—	4,000	7,949	329	316	416	540	298
Rats	42,969	—	502	1,294	931	969	207	317	54

Mean specific Cr^{51} activity of organs (cpm/gm tissue [illegible] rats, 16 mice in each group)
Normal = non-splenectomized animals
Splx = splenectomized animals

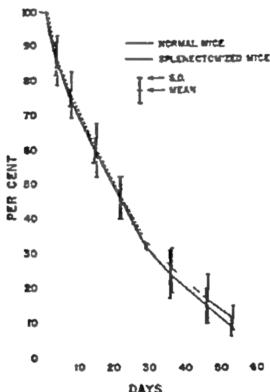


Fig. 1. Survival of Cr^{51}-labeled erythrocytes in mice

The organ distribution of the radioactivity in the mice sacrificed when the red cell life span reached the 10% level showed high activity in the spleen (14.4% of administered dose) and in the liver (11.6%). In the other organs, only very low activity was found, totaling an additional 2% (fig. 2). The specific activity of the spleen

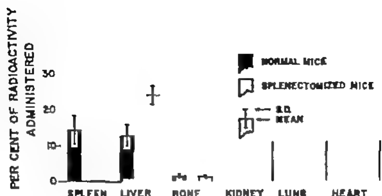


Fig 2. Organ distribution of Cr^{51}-labeled erythrocytes in mice.

was thirteen times greater than that of the liver (table I) After splenectomy the total radioactivity (fig 2) and the specific activity (table I) of the liver doubled. The bone marrow kidneys, lungs and heart demonstrated no change (fig 2) The total radioactivity of spleen and liver in normal mice (26 / of the administered dose) was only slightly greater than the radioactivity of the liver in the splenectomized mice (23 /)

Rats In contrast to the observation in mice, the T/2 Cr^{51} Rbc in the normal rats was 18.2±2 1 (S.D) days, whereas in the splenectomized rats the T/2 Cr^{51} Rbc was 21 0±2 69 days, a small but significant increase (p <0 01) in life span (fig 3) The mean erythrocyte survival approximated from the life span curves was 58 and 60 days in normal and splenectomized rats, respectively

In the normal rat, the mean total radioactivity in the spleen was 22 / of the administered radioactivity four times greater than that found in the liver Radioactivity in bone marrow was 4.5 / of the administered dose or about equal to that of the liver (fig 4) The specific activity of the spleen was 86 times greater than that of the liver (table I) In the splenectomized rats, the liver radioactivity increased about two-and-a half-fold and the bone marrow two-fold compared to the normal animals the other organs remained unchanged. Total radioactivity in spleen, liver and 'bone marrow' was 31 / of the administered dose in the normal rats, whereas post splenectomy the total radioactivity of liver and 'bone marrow' was only 21 /.

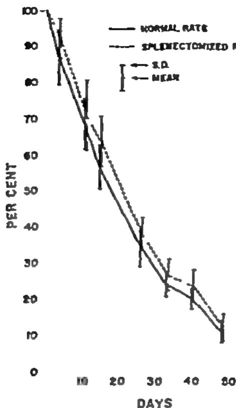


Fig 3. Survival of Cr^{51}-labeled erythrocytes in rats.

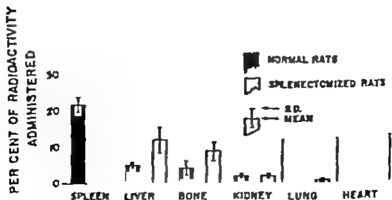


Fig 4 Organ distribution of Cr^{51}-labeled erythrocytes in rats.

Duration

In our study T/2 Cr^{51} Rbc in mice was found to be approxi-
ely 20.2 days, a finding comparable to reports in the literature
15, 16) GOODMAN found a variation in the half life from 15 to
lays depending on the strain in the DBA/2 mice, the 50 % level
19 7 days (limits 18.5 to 20 8) with an average erythrocyte life
1 of 50—55 days (14) Our experiments suggest that in mice
h spleen and liver participate in the removal of aged red cells
that splenectomy does not change the life span of the erythro-
s this can be explained by the fact that the liver has the
acity to double its erythrocyte sequestration, taking over comp-
ly the role of the spleen.

Table II
Red cell life span in normal rats.

Investigator	Reference	T/2 Cr^{51} Rbc
DONOHUE et al. 1953	17	10.0
GOLLETT et al. 1956	20	18.4
HALL et al. 1957	9	19.9
BELCHER AND HARRIS 1958	12	20.7
SMITH et al. 1959	[illegible]	18.5
GOODMAN AND SMITH 1961	14	19.6
TIZIANELLO et al. 1961	10	12.2
THOMPSON et al. 1961	11	18.0
HUGHES JONES AND CHENEY 1961	19	18.0
AZIZ AND SCHILLING 1964	18	13.2
ULTMANN AND GORDON		18.2

The results of studies of Cr^{51}-labeled red cell life span in rats, as
blished in the literature, are summarized in table II The T/2
^{51} Rbc of the rat was found to be about 18.2 days in our studies,
ich is in good agreement with seven of the ten published measure
ents. THOMPSON et al. (11) have suggested that the shorter red cell
e span obtained by some investigators (10 17 18) may possibly
due to use of Cr^{51} of low specific activity or to Bartonella infesta
n, but more likely is due to lack of uniform immunologic charac
ristics in so-called in bred strains of rats. HUGHES JONES AND
HENEY (19) have shown that, in the normal rat, the spleen destroys
out 60 % of labeled red cells, the liver about 10 %, and the bone

marrow 5 to 10 %. Comparing their report (Ref. 19 fig 5) with ours, the data are almost identical indicating that approximately 25 % of the injected Cr^{51} is found in the spleen on day 55. HALL et al. (9), THOMPSON et al. (11), BELCHER AND HARRISS (12) and TIZIANELLO et al. (10) studied the effect of splenectomy on the red blood cell life span in rats. According to HALL et al. (9) and TIZIANELLO et al. (10) the removal of the spleen shortens the erythrocyte life span to 8 and 7 days, respectively. Our study of 18 splenectomized rats showed a small but statistically significant prolongation of erythrocyte life span compared to the normal rats. THOMPSON et al. (11) who employed Cr^{51} found the T/2 Cr^{51} Rbc prolonged by approximately 2.5 days. BELCHER AND HARRISS (12) who used Fe^{59} in these studies, also found a prolonged erythrocyte life span in splenectomized rats. Although the difference in the red cell life span in normal and splenectomized animals is not very dramatic, the organs reflect the cumulative degree of changes in sequestration pattern. In the splenectomized rats, the liver and the bone marrow double their sequestration of the labeled erythrocytes; however, their combined increased sequestrating capacity does not approach that of spleen, liver and bone marrow of the normal animals. The increase in the red cell life span in the splenectomized rats is explained by these findings. This is markedly different from the effect of splenectomy in mice, in which the life span of the erythrocytes is not changed following splenectomy because the liver sequestrates all the red cells formerly sequestered by the spleen.

The finding of increased bone marrow sequestration of normal red cells in splenectomized rats is of particular interest in that it emphasizes the differences in sites of erythrocyte destruction in various species. In the mouse, the bone marrow plays a minor role in erythrocyte destruction in the normal and in the splenectomized animal. In the normal rat, the reticuloendothelial cells of the marrow contribute only slightly to erythrocyte destruction; however, after splenectomy, the activity of marrow sequestration doubles. In contrast to the findings in mice and rats are MITSCHLER's (7) and VON EHRENSTEIN's (8) observations in the rabbit where the bone marrow appears to be the chief site of red cell destruction, responsible for over 50 % of normal red cell destruction.

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Summary

Normal, Cr^{51}-labeled erythrocytes of mice or rats were studied in normal and splenectomized animals to determine red cell life span and sites of sequestration. In mice, the T/2 Cr^{51} Rbc is 20 days in normal as well as splenectomized animals. In mice, hepatic sequestration doubles following splenectomy removing all the erythrocytes formerly found in the spleen. In splenectomized rats, the T/2 Cr^{51} Rbc is slightly longer (21 days) than in the normal rats (18 days). In the splenectomized animals, liver and bone marrow sequestration double but are nevertheless only 2/3 of combined spleen, liver and bone marrow sequestration found in the normal rats. The observation that splenic sequestration is the major site of normal erythrocyte destruction in mice and rats contrasts with the findings in rabbits in which the bone marrow appears to be the chief site of red cell destruction.

Résumé

Chez des souris et des rats à l'état normal, et après splénectomie, le temps de vie et les lieux de séquestration d'érythrocytes normaux marqués au Cr^{51} ont été étudiés. Chez la souris, le T/2 Cr^{51} des érythrocytes, est de 20 jours à l'état normal et après splénectomie, la séquestration hépatique est doublée après splénectomie, englobant tous les érythrocytes qui auparavaient se trouvaient dans la rate.

Chez les rats le T/2 Cr^{51} des érythrocytes est, après splénectomie, un peu plus long (21 jours) que chez les rats normaux (18 jours). La séquestration dans le foie et la moelle osseuse est doublée, mais en représente pas plus que 2/3 de celle combinée de la rate du foie et de la moelle osseuse trouvée chez des rats normaux. L'observation que la séquestration splénique est l'endroit le plus important de la destruction des érythrocytes normaux chez la souris et le rat, contraste avec les constatations faites chez le lapin qui démontrent que la moëlle osseuse est le lieu principal de la destruction des érythrocytes.

Zusammenfassung

Bei normalen und splenektomierten Mäusen und Ratten wurden die Lebensdauer normaler mit Cr^{51} markierter Erythrozyten, sowie der Ort ihrer Sequestration bestimmt. Bei normalen und bei splenektomierten Mäusen beträgt T/2 Cr^{51} RBK 20 Tage. [illegible] Sequestration in der Leber verdoppelt sich bei der Maus nach Splenektomie [illegible] alle Erythrozyten entfernt werden, die zuvor in der Milz nachzuweisen [illegible] splenektomierten Ratten ist T/2 Cr^{51}-RBK etwas länger (21 Tage) als bei [illegible] tieren (18 Tage). Bei splenektomierten Tieren verdoppelt sich die Sequestration [illegible] Leber und Knochenmark, sie beträgt aber trotzdem nur zwei Drittel der [illegible] Sequestration in Milz, Leber und Knochenmark normaler Ratten. Die [illegible] wonach bei Mäusen und Ratten der Erythrozytenabbau vorwiegend [illegible] findet, steht im Gegensatz zu den Befunden bei Kaninchen, wo das [illegible] die hauptsächliche Stätte der Erythrozytendestruktion darstellt.

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Varia

Deutsche Hämatologische Gesellschaft

Die 11. Tagung der Deutschen Hämatologengesellschaft findet vom 13.–16. Oktober 1965 unter dem Vorsitz von Prof. Dr. H. Braunsteiner in Innsbruck statt. Die folgenden Themen stehen auf dem Programm: 1. Struktur normaler und abnormer Gamma-Globuline. 2. Lymphocytenfunktion. 3. Strahlenhämatologie. 4. Chemotherapie mit Vinca-Rosea-Alkaloiden. 5. Basophile und Mastzellen. 6. Moderne hämatologische Methoden.

Die gleichzeitig stattfindende Tagung der Gerinnungssektion steht unter dem Vorsitz von Prof. Dr. E. Deutsch (Wien) und hat die Reinigung und therapeutische Anwendung von Gerinnungsfaktoren zum Thema.

Neben den Hauptthemen werden im Rahmen der zeitlichen Möglichkeit Mitteilungen aus allen Gebieten der Hämatologie angenommen. Es wird ersucht, Anfragen und Anmeldungen an Prof. Dr. H. Braunsteiner, Medizinische Universitätsklinik Innsbruck, zu richten.

Libri

Lennert, K.: **Pathologie der Halslymphknoten.** *Springer-Verlag, Berlin 1964.* VIII + 128 S., 65 Abb., Preis DM 26.–

Prof. K. Lennert hat sich, von der Haematologie herkommend, seit Jahren mit der Lymphknotenpathologie befaßt. Die vorliegende kleine Monographie ist das Resultat des für die Deutsche Onkologen-Tagung 1963 erstatteten Referates über die Pathologie der Halslymphknoten. Nach einleitenden technischen Vorbemerkungen wird die Morphologie der einzelnen Erkrankungen der Halslymphknoten in prägnanter Form abgehandelt. Zytologische und histologische Kriterien werden dabei in glücklicher Weise kombiniert. Gleichzeitig werden die dazugehörigen Blutbildveränderungen diskutiert. Besonders wertvoll wird die Monographie dadurch, daß nicht nur die im Band I/3 des Handbuches der speziellen Pathologie von Henke-Lubarsch-Rössle ausführlich dargestellten entzündlichen Lymphknotenerkrankungen besprochen werden, sondern auch die neoplastischen Lymphknotenaffektionen. Auch auf diesem schwierigen Gebiet verfügt der Verfasser über große eigene Erfahrung, die es ihm gestattet, die praktisch wichtigen Gesichtspunkte hervorzuheben, unter Vermeidung weitschweifiger theoretischer Erörterungen. Die vorliegende Monographie wird zweifellos dem in der Diagnostik tätigen Pathologen und Haematologen ein unentbehrlicher Ratgeber sein. Sie ist aber auch dem Kliniker und dem praktischen Arzt, der sich rasch über die morphologische Lymphknotendiagnostik orientieren will, sehr zu empfehlen. Zahlreiche gute Abbildungen illustrieren den Text.

F. Gloor, Basel

II *A. D. Anderson:* Synopsis of Pathology. 6. edition. C. V. Mosby Co., St. Louis 1964. 653 p., Price $ 9.75.

The volume mainly designed for the medical student and the practicing physician is a good short presentation of general and special pathology. The chapters on the reticuloendothelial system, spleen and lymphnodes and the blood forming organs are informative.

Each chapter has a list of selected references. G. Rosenow, New York

A. G. Steinberg/A. Bearn: Progress in Medical Genetics Vol. 3. Grune & Stratton, New York, N. Y. 1964. 272 p., Price $ 12.25.

The first chapter of this volume, by B. S. Strauss, Chemicals and Mutagens and the Genetic Code is a penetrating and critical review not only of mutagenesis, but of much of the whole structure of molecular genetics, particularly as related to the coding problem. The next four chapters deal with more specific human genetic aspects: Pharmacogenetics by A. G. Motulsky, Genetical Variants of Human Serumcholinesterase by H. Lehmann and J. Liddell, Transplantation Antigens in Mouse, Rat and Man by B. Amos, and The 21 Trisomy-Current Stage of Chromosomal Research by J. Lejeune. (21 trisomy is the author's term for Down's syndrome or mongolism.) There follows Chapter 6 on Multifactorial Inheritance and Human Disease by J. A. Fraser Roberts and finally The Quantitative Assessment of Hereditary Damage Induced by Radiation by A. P. James and H. B. Newcombe.

There is an excellent balance in the reviews between general discussions as those by Strauss and Fraser Roberts, and detailed treatments of specific genetic variations involving medically important phenomena as, for example, those by Motulsky and Lejeune. This volume will be useful immediately and in spite of the rapid progress in medical genetics will serve as a source of knowledge for years to come.

Curt Stern, Berkeley

Acta haemat. 33: 129–138 (1965).

Department of Pediatrics of the University of Pavia

The Mechanism of Haemolysis in Favism

Some Analogy in the Activity of Primaquine and Fava Juice

By Franco Panizon and Franco Zacchello

Although the agreement on this opinion is not complete, some definite observations strongly suggest a similar pathogenesis for the haemolysis in favism and in primaquine sensitivity. Subjects of the Mediterranean area who are sensitive to fava beans show in their erythrocytes some biochemical features very similar to those of primaquine-sensitive Negro people: they are known to be sensitive also to primaquine (14, 20, 22, 25), while the American Negroes are apparently insensitive to fava beans (4). Moreover, during the haemolytic crisis induced by fava beans Heinz bodies appear in red cells just as they do during the primaquine induced haemolytic crisis.

Several authors, using primaquine or related substances like acetylphenylhydrazine, have been able to induce in red cells some peculiar and well reproducible effects, while less definite results have been obtained using fava extracts.

Primaquine in vitro induces a decrease of reduced glutathione (GSH) (1), ATP (16) and inorganic pyrophosphatase (5, 19) in glucose-6-phosphate dehydrogenase (G6PD) deficient erythrocytes. In both normal and deficient erythrocytes it induces increase in osmotic fragility (26, 27), loss of potassium (26, 27), enhancement of anaerobic glycolysis correlated with a depression of the aerobic one (23), and the appearence of methaemoglobin and of Heinz bodies (2, 11). Conversely, fava extracts show a similar influence only on the anaerobic glycolysis (23) and on the GSH stability.

The latter effect was firstly found by Mela and Perona (15) who demonstrated that incubation with fava beans induces a

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decrease in sensitive red cells. Bowman and Walker (5) while confirming this finding, reported that the effect of fava beans was more rapid and less pronounced than that of acethylphenylhydrazine, and that it was evident also in normal erythrocytes although in a lesser degree. Than Contu et al. (7) noted an almost identical decrease of GSH in both normal and deficient red cells. Szeinberg et al. (24) were not able to observe any fall of GSH using saline extracts of dried beans. The inconsistent results of the experiments with fava beans might be due to the quantity of active substance present in the extracts, which is small in comparison to the relatively high doses of primaquine or similar drugs commonly employed. In vivo the quantity of fava beans required to induce an haemolytic crisis is actually far larger than that of primaquine and can be tentatively estimated to be 10 50 000 times greater (17). In this paper we report the results of a comparative study of the effects of fava juice and primaquine on the GSH stability, Heinz bodies appearence, methaemogloban formation, osmotic fragility and potassium loss in normal and sensitive red cells. In addition differential survival of red cells treated in vitro was studied by injecting Cr^{51}-labeled red cells in normal recipients.

Materials

Blood. Donors where adult Italian males, both normal and G6PD-deficient who have never had clinical signs of favism nor evidence of other haematological disorders. Heparinized blood, added with glucose (2 mg/ml) was kept in refrigerator and tested within 24 hours.

Fava juice and fava extract. Fava juice obtained by squeezing the beans in an hydraulic press at 400 atmospheres was subsequently cleared by centrifugation.

Fava extracts were obtained by homogenizing the fava beans, added with one volume distilled water in a blendor, clearing by filtration through paper, lyophilizing and finally dissolving again in one volume.

For the experiments on potassium loss and osmotic fragility the juice or extract was dejonized and subsequently was made iso-osmotic with sodium chloride.

For transfusional experiments the fava juice was sterilized by filtration through Seitz's filter.

Primaquine. The substance was dissolved in saline solution buffered at pH 7.4 and then sterilized, for transfusional experiments, by heat at 100 °C.

Methods

GSH was determined according to Stevenson et al. (21), methaemoglobin following Evelyn and Malloy (8), osmotic fragility according to Parpart (16)-

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They are born and living in a small Italian district, the Delta of Po river, where malaria was endemic until forty years ago, and where it is actually an important insulate of Thalassemia and a minor one of G6PD-deficiency.

Staining of Heinz bodies was done with cristal violet. The loss of potassium from red cells was evaluated through the potassium increase in serum, measured by flame fotometer (EEL). For transfusion experiments erythrocytes were labeled with Cr^{51}, washed and resuspended in sterile saline, and transfused without adding ascorbic acid. The blood radioactivity was measured by well type scintillation counter. Haematocrit readings were not performed.

Experiments

In vitro experiments with intact erythrocytes: Whole blood was incubated for 3 hours at 37 °C with added alternatively fava juice or extract to final concentration of 50‰, or primaquine to a final concentration of 0.002–0.050%. Experiments have been carried out with both normal and G6PD-deficient erythrocytes. Control samples were incubated without any addition but glucose.

At the end of the incubation period, in both test and control samples the following determinations were done: GSH and methaemoglobin content of the red cells; potassium concentration in the serum; osmotic fragility of the red cells; staining for Heinz bodies.

In vitro experiments with haemolyzed blood: Whole blood, haemolyzed by adding saponine, was incubated at room temperature for 15 minutes with added alternatively fava juice, primaquine, or acetylphenylhydrazine. Control samples were incubated without any addition.

At the end of the incubation period, in both test and control samples the GSH content was measured.

This kind of experiments was carried out with normal blood only, for at the end of the incubation time G6PD-deficient haemolyzed blood would show too low GSH values to give reliable data.

Transfusion experiments: Both normal and G6PD-deficient erythrocytes have been incubated, as said above, with fava juice, primaquine 0.002%, primaquine 0.010%. Control samples have been incubated without any addition. After 3 hours incubation, red cells have been tagged with Cr^{51}, washed and transfused to normal receptors, compatible for the ABO and Rh system. Blood was taken from the recipients 30 minutes after transfusion and then at regular intervals for 96 hours, in order to calculate the rate of the disappearance of radioactivity.

Results

GSH stability. In intact G6PD-deficient erythrocytes the incubation with 0.002–0.020% primaquine induces a definite decrease of the GSH content, proportional to the dose. This decrease is uniformly progressive. No modification at all was observed in normal erythrocytes. Fava juice or extract also induce a decrease of GSH in deficient erythrocytes, similar to that induced by 0.002% primaquine. The decrease is similarly progressive. No GSH decrease was observed in normal erythrocytes (table I). On the contrary in haemolyzed blood the GSH fall takes place much more quickly after incubation with fava juice than after incubation with primaquine or acetylphenylhydrazine; besides this effect is still well appreciable in normal blood (table II).

Table I

Fall of GSH in normal and sensitive intact erythrocytes incubated with either primaquine or fava extract or juice

Substance added		Normal RBC		G6PD-deficient RBC		
		No. of obs.	GSH fall, mg%/ml RBC after 3 h	No. of obs.	GSH fall, mg%/ml RBC after 30'	after 3 h
Primaquine	0.002%	[illegible]	0 ± 2	6	1 ± 1	13 ± 3
	0.005%	6	0 ± 4	6	5 ± 3	22 ± 3
	0.010%	6	1 ± 2	6	10 ± 2	26 ± [illegible]
	0.020%	[illegible]	1 ± 3	6	14 ± 3	29 ± 6
Fava	extract	12	0 ± 5	5	4 ± 4	12 ± 4
	juice	10	0 ± 5	9	6 ± 4	11 ± 3

Table II

Fall of GSH in normal haemolyzed blood incubated during 15 minutes with acetylphenylhydrazine, primaquine fava extract and fava juice

Substance added		No. of obs.	GSH fall mg%/ml blood
Acetylphenylhydrazine	0.500%	4	1 ± 1
Primaquine	0.200%	4	5 ± 2
Fava extract		4	13 ± 7
Fava juice		4	32 ± 5

Methaemoglobin and Heinz body formation. Both normal and G6PD-deficient erythrocytes, incubated with primaquine, show methaemoglobin formation proportional to the drug concentration. At concentrations lower than 0.005% no methaemoglobin production was found. Heinz bodies have never been observed. Incubation with fava juice does not induce either methaemoglobin production or Heinz bodies appaerence (table III).

Table III

Formation of methaemoglobin (MHB) in normal and sensitive erythrocytes incubated during 3 hours with primaquine, fava extract and fava juice.

Substance added		Normal RBC		G6PD-deficient RBC	
		No. of obs.	MHb, % of total Hb	No. of obs.	MHb, % of total Hb
Primaquine	0.002%	7	<3	6	3 ± 2
	0.005	7	5 ± 2	6	9 ± 3
	0.010%	7	13 ± 4	6	18 ± [illegible]
	0.020%	7	23 ± 7	6	[illegible] ± 6
Fava	extract	12	<3	17	<3
	juice	10	<3	15	<3

Loss of potassium. Incubation with primaquine above the 0 005 % concentration induces a relevant potassium loss both from normal and deficient erythrocytes. No similar effects was noted after incubation with fava juice (table IV)

Table IV
Potassium loss from normal and sensitive erythrocytes incubated with either primaquine or fava juice

Substance added		Normal RBC		G6PD-deficient RBC	
		No. of obs.	K loss, mEq/l RBC	No. of obs.	K loss, mEq/l RBC
Primaquine	0.002%	8	0.3 ± 0.5	8	0.3 ± 0.4
	0.005%	8	1 1 ± 0.9	8	0.7 ± 0.5
	0.010%	8	2.3 ± 1 1	8	1.7 ± 1.0
	0.020%	8	4 7 ± 1 7	8	5.2 ± 1.3
Fava juice		8	0 0 ± 1.0	8	0.0 ± 1.0

Osmotic fragility Incubation with primaquine results in an increase of osmotic fragility proportional to the drug concentration. This effect is more evident in normal than in G6PD-deficient erythrocytes. The lowest useful concentration of primaquine is 0.005% for the normal and 0 010° for the defective red cells. Incubation with fava juice increases osmotic fragility in both normal and defective erythrocytes, without any difference between the kinds of blood The increase in cellular fragility is of the same order as that induced by the highest concentration of primaquine (0.050%) (table V)

In vivo survival of pre-incubated erythrocytes G6PD-deficient erythrocytes, incubated for 3 hours with primaquine, undergo, when

Table V
Increase of haemolysis rate in 0.45% saline of normal and sensitive erythrocytes after 3 hours incubation with either primaquine of fava juice.

Substance added		Normal RBC		G6PD-deficient RBC	
		No. of obs.	Increase of haemolysis,	No. of obs.	Increase of haemolysis, %
Primaquine	0.002%	8	0.2 ± 1.2	8	0.2 ± 1.0
	0.003%	8	2.6 ± 1 4	8	0.3 ± [illegible]
	0.010%	8	6.0 ± 2.3	8	1 1 ± 1.5
	0.020%	8	12.3 ± 2.6	8	4.9 ± 1.7
	0.050%	8	33.4 ± 3.9	[illegible]	12.0 ± 3.1
Fava juice		6	36.6 ± 3 4	8	29 1 ± 3.2

recipients. The first group of effects occours both in G6PD-deficient and in normal blood, and only at relatively high concentrations of primaquine the latter in the deficient blood only at even the lowest primaquine concentration. The diminution of GSH induced by fava juice in G6PD-deficient erythrocytes was found in our experiments to be very constant and highly characteristic, and to take place in a progressive manner like the diminution induced by primaquine. Furthermore, we have observed that fava juice added to normal haemolyzed blood results in a very rapid decrease of GSH. This effect explains the results obtained by BOWMAN AND WALKER (3) and by CONTU et al. (7) who have observed a very quick GSH fall even in normal erythrocytes. These results could be due to the fact that the first step of the method of GRUENERT AND PHILLIPS (10) (used by the above authors to measure GSH) results in haemolysis of the blood sample. In this step, whose duration, although no definitely stated, could reach 15 minutes following FLANAGAN et al. (9) fava juice can induce a considerable GSH fall, unrelated to the real effects of the previous incubation. The impairment of the capacity of G6PD-deficient erythrocytes to survive in vivo after "in vitro incubation with primaquine or fava juice, seems to be strictly related to the fall in their GSH concentration and suggests a kind of action common for both fava juice an primaquine.

In conclusion, our data strongly support the hypothesis that in Mediterranean G6PD-deficient people, fava beans and primaquine act through a similar mechanism in determining haemolysis, and seems to rule out the hypothesis of an allergic pathogenesis for favism that a few authors (6, 12, 13) still emphasize.

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Summary

Fava juice or extracts induce fall of reduced glutathione (GSH) and definite impairment of the survival of G6PD-deficient erythrocytes. Normal erythrocytes remain unaffected. This effects are very similar to those induced by primaquine at low concentration. An explanation is given for different results concerning GSH stability reported in the literature. Some effects induced in both normal and G6PD-deficient erythrocytes by higher primaquine concentrations, as methaemoglobin formation and potassium loss, cannot be reproduced t all with fava juice. A difference is found between primaquine and fava juice in regard to their effect on the erythrocyte osmotic fragility. A similar kind of action in determining haemolysis is supposed for primaquine and fava beans.

Résumé

Le suc ou des extraits de fèves provoquent une chute du glutathion (GSH) réduit et une diminution de la survie des érythrocytes déficients en G6PD. Les érythrocytes normaux ne sont pas touchés. Les effets sont très semblables à ceux provoqués par la primaquine à de basses concentrations. Une explication est donnée pour la divergence des résultats concernant la stabilité du GSH, rapportés dans la littérature. Quelques effets produits, aussi bien dans des érythrocytes normaux que dans des érythrocytes déficients en G6PD par des concentrations de primaquine plus élevées, tels que la formation de methémoglobine et la perte de potasse, ne peuvent pas du tout être reproduits par le suc de fèves. La primaquine et le suc de fèves influencent de façon légèrement différente la résistance osmotique des érythrocytes. La façon dont est déterminée l'hémolyse est supposée être semblable pour la primaquine et les fèves.

Zusammenfassung

Der Saft oder Extrakte von Favabohnen rufen eine Abnahme von reduziertem Glutathion (GSH) und eine Verkürzung der Lebensdauer roter Blutkörperchen mit einem G6PD-Mangel hervor. Dieser Effekt gleicht weitgehend der Wirkung von Primaquin in niedrigen Konzentrationen. Es wird eine Erklärung für die abweichenden Angaben der Literatur über die GSH-Stabilität gegeben. Gewisse Einflüsse höherer Primaquinkonzentrationen auf normale und G6PD-Mangel-Erythrozyten, wie Methämoglobinbildung und Kaliumverlust, lassen sich mit dem Saft der Favabohnen nicht reproduzieren. Zwischen Primaquin und Favabohnensaft besteht ein gewisser Unterschied mit Bezug auf die Beeinflussung der osmotischen Erythrozytenresistenz. Es wird vermutet, daß den hämolysierenden Wirkungen von Primaquin und Favabohnen derselbe Mechanismus zu Grunde liegt.

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Aus dem Institut für experimentelle Krebsforschung (Direktor: Prof. A. Graffi)
der Deutschen Akademie der Wissenschaften zu Berlin, Berlin-Buch

Untersuchungen zur hämatologischen Aufsplitterung der durch das Virus der myeloischen Leukämie der Maus induzierten Leukosen*

Von F. Fey und A. Graffi

Das aus den transplantablen Mäusetumoren Sa I, Sa II (Landschütz) und dem Ehrlich-Ascitescarcinom isolierte Virus induzierte nach Applikation in neugeborene Mäuse des in Ruhe domzucht gehaltenen Stammes Agnes Bluhm zu 50 bis 75% ursprünglich fast ausnahmslos myeloische Leukämien (9, 11, 8, 2). Von den nach einer durchschnittlichen Latenzzeit von 31 Wochen auftretenden Leukämien zeigten über 70% der Tiere eine für die Chloroleukämie charakteristische grüne Verfärbung des leukämischen Gewebes (9, 10, 11, 8, 2). Nachdem wir das leukämische Gewebe virusinduzierter Leukämien zur Herstellung zellfreier Filtrate in aufeinanderfolgenden Passagen verwendeten, konnten wir eine Aktivitätssteigerung des Virus hervorrufen, die sich in einer merklichen Erhöhung der Leukämiequote und einer wesentlichen Verkürzung der Latenzzeit ausdrückte (12). Im Verlauf dieser Versuche beobachteten wir eine Abnahme des Chloroleukämie-Vorkommens, die wir zuvor bei der Verwendung zellfreier Filtrate aus einer transplantablen Leukose (SOV 16) schon bemerkt hatten (12). Mit dem aktivierten Virus konnten heterolog bei Ratten Leukämien in beachtlicher Zahl induziert werden (7), deren Leukämiefiltrate bei Mäusen in hohem Prozentsatz Leukämien unterschiedlicher hämatologischer Typen hervorriefen (3, 13). Eine ähnliche Aufspaltung stellten wir in der Folge auch bei den homologen Übertragungsversuchen mit zellfreiem Leukämiematerial fest (4). Interessanterweise scheint sich bei dem Gross'schen Agens,

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* Herrn Prof. Dr. H. Hamperl zur Vollendung des 65. Lebensjahres gewidmet.

das primär ausschließlich lympathische Leukämien induzierte eine ähnliche Tendenz zur Aufsplitterung in unterschiedliche Leukämietypen bemerkbar zu machen (17)

Der Mechanismus, der zur Aufspaltung der zuvor einheitlichen Leukämieformen führt ist weitgehend unbekannt. Als Faktoren, die eventuell eine derartige Änderung bewirken, können die genetische Konstitution der Empfängertiere, eine Abwandlung der Histo-Spezifität des Virus und eine bestimmte Selektionierung von Virusmutanten in Betracht gezogen werden. Weiterhin wäre zu klären, ob der hämatologische Typ der Leukämie, aus dem das Filtrat bereitet wird, einen Einfluß auf die Ausprägung der induzierten Leukämien ausübt.

Unsere nachfolgend beschriebenen Versuche sollen einen Beitrag zur Aufklärung dieser Problematik darstellen. Wir halten es für notwendig die Klassifizierung der in unseren Versuchen aufgetretenen Leukämietypen mit ihren charakteristischen Merkmalen zu umreißen, da nach unserer Auffassung mit einer sorgfältigen Diagnostizierung und Typisierung der Leukämien möglicherweise viele in der Literatur aufgetretene Differenzen in der Befundinterpretation beseitigt werden könnten.

Material und Methoden

Tiermaterial. Es wurden Mäuse der in Randomzucht gehaltenen Inzuchtstämme Agnes Bluhm (AB) XVII/Bln und in wenigen Fällen CBA/Bln verwendet.

Filtratbereitung Lymphknoten, Thymus sowie Anteile von Milzen und Lebern leukämischer Tiere wurden in der von uns beschriebenen Weise (17) aufgearbeitet. Das zellfreie Filtrat wurde in der Verdünnung 1 10 in einer Dosis von 0,1 ml neugeborenen Mäusen der erwähnten Stämme subkutan appliziert.

Diagnostizierung Nach Manifestwerden der Leukämie wurden die Tiere getötet, nachdem vorher Blutausstriche angefertigt und die Leukozytenzählung durchgeführt wurde. Von jeder leukämischen Maus wurden die Blutausstriche und Tupfpräparate des Knochenmarks, der Milz und der Leber mit Giemsa gefärbt und Paraffinschnitte von Sternum, Lymphknoten, Thymus, Milz und Leber angefertigt. Weiterhin wurde bei einem Teil der Tiere zytochemisch die alkalische und saure Phosphatase- und die unspezifische Esterase-Aktivität mit Hilfe der Azokupplungstechnik an Blutausstrichen und Kryostatschnitten der obenerwähnten Organe bestimmt (4 5)

Ergebnisse

Wir haben über 400 Fälle der durch das Myelosevirus induzierten Leukämien auf Grund des hämatologischen, histologischen und zytochemischen Befundes in acht Haupttypen eingeteilt. Nachfolgend soll eine kurze Charakteristik der betreffenden hämatolo-

gisch differenten Leukämieformen den Resultaten, die sich aus den Filtratversuchen ergaben, vorangestellt werden

Myeloische differenzierte Leukämien (Abb 1)

Diese Leukämieform zeichnet sich durch das Vorherrschen differenzierter neutrophiler Granulocyten aus, i. e. reifer und jugendlicher ringkerniger Zellen. Die Anzahl der Leukämiezellen im peripheren Blut schwankt, im allgemeinen liegt der Wert zwischen 50–100000. Häufig kommen Mitoseanomalien vor die in Form von Pseudo-Pelgerzellen in Erscheinung treten. Diese Zellen sind im Gegensatz zu den echten Pelgerzellen zweikernig und folglich tetraploid. Bei dieser Leukämieform findet sich eine merkliche Anisocytose und Anisonukleose. Im allgemeinen tritt die differenziert zellige myeloische Leukämie generalisiert auf, in einigen Fällen konnten auch intrathorakal lokalisierte weitgehend reifzellige myeloische Leukosen nachgewiesen werden. Zytochemisch ist eine starke Peroxydase-Aktivität zu vermerken; alkalische und saure Phosphatase sind negativ Die unspezifische Esterase ist schwach positiv

Tabelle I

Aufspaltung in hämatologisch differente Leukämietypen. Filtrate von myeloischen differenzierten Leukämien.

Leukämietyp	Fälle	Prozentsatz	Mäusestamm XVII	AB	CBA	Latenzzeit in Wochen
Myel. (differenz.) Leukämien	22	30,5	7	13	2	15,0
Unreife myeloische Leukämien	12	16,7	6	6		13,7
Chloro-Leukämien	23	31,9		20	3	18,0
Paramyeloblasten-Leukämien	7	9,7	2	5		13,1
Myeloisch-retikul. Leukämien	6	8,4		5	1	15,1
Retikuläre Leukämien	2	~2,8		2		11,5
Retikulär-lymphat. Leukämien	—					
Lymphatische Leukämien	—					

Die nach Injektion von Filtraten myeloischer differenzierter Leukämien bei Mäusen auftretenden Leukämieformen zeigt Tabelle I. Wie ersichtlich ist, gehören ca. 90% der entstandenen Leukämien der myeloischen Reihe an. Lediglich eine kleine Tendenz zur Differenzierung in retikulärer Richtung ist zu verspüren. Auffallend ist der hohe Anteil an Chloroleukämien bei AB-Mäusen und das gänzliche Fehlen bei XVII Mäusen.

Unreifzellige myeloische Leukämien (Abb 2)

Diese Leukämieform ist in ihrer Zusammensetzung sehr heterogen. Die Prädominanz verschiedener Zelltypen, die unter dem Überbegriff „unreifzellige" zusammengefaßt sind, ist ein Charakteristikum der unreifzelligen Leukämie. Die unreifsten Formen stellen die Parapromyelocyten dar die als größte myeloische Blutzellen im Ausstrich auftreten. Weiterhin kommen die Paraformen der Myelocyten vor und da-

neben jugendliche und ausgereifte Granulozyten in wechselnder Menge. Eine weitere häufig anzutreffende Leukämieform setzt sich größtenteils aus leukämischen Myelozyten und jugendlichen Neutrophilen zusammen, die Promyelozyten treten in diesen Fällen zurück. Differentialdiagnostisch ist diese Leukämieform mitunter anhand des Blutausstriches nicht mit Sicherheit von einer leukämoiden Reaktion zu unterscheiden. Zur Sicherung des Befundes müssen dann histologische Kriterien herangezogen werden. Die Leukämiezellen treten meist in beträchtlicher Anzahl in der Peripherie auf, ihre Werte übersteigen meist 100000. Wie bei den differenzierterzelligen myeloischen Leukämien treten auch hier und zwar in erhöhtem Maße intrathorakal lokalisierte Leukosen auf, wobei die Anzahl der Leukämiezellen im Blut äußerst gering sein kann. Zytochemisch läßt sich Peroxydase zum Teil in den Parapromyelozyten und mit deutlicher Aktivität in den reiferen Stadien nachweisen. Eine ebenfalls deutliche Reaktion auf alkalische Phosphatase ist bei den Parapromyelozyten zu bemerken, während die reiferen Zellen negativ reagieren. Die saure Phosphatase ist negativ, die unspezifische Esterase schwach positiv.

Tabelle II

Aufspaltung in hämatologisch differente Leukämietypen. Filtrate von unreifzelligen myeloischen Leukämien.

	Fälle	Prozentsatz	Mäusestamm		Latenzzeit in Wochen
			XVII	AB	
Myel. (differenz.) Leukämien	6	18,8	4	2	14,5
Unreifz. myeloische Leukämien	6	18,8	5	1	16,1
Chloro-Leukämien	4	1..5	3	1	21,2
Paramyeloblasten-Leukämien	5	15,6	5		13,2
Myeloisch-retikuläre Leukämien	9	28,1	5	4	14..
Retikuläre Leukämien	1	~3,1	1		12,0
Retikulär-lymphatische Leukämien	1	~3,1		1	19,0
Lymphatische Leukämien	—				

Filtrate aus unreifzelligen myeloischen Leukämien induzierten die in Tabelle II angeführten Leukämieformen. Es ist zu ersehen, daß die Aufspaltungstendenz der unreifzelligen myeloischen Leukämien recht erheblich ist. Nur noch ca. zwei Drittel der entstehenden Leukämien gehören der myeloischen Reihe an, davon sind ein Großteil unreifzellig. Auffallend ist der hohe Prozentsatz an myeloisch-retikulären Mischformen.

Chloroleukämien (Abb. 3)

Durch die charakteristische grüne Verfärbung des leukämischen Gewebes ist diese Leukämieform als recht einheitlich zu bezeichnen. Bezüglich der Zellpopulation wird jedoch ersichtlich, daß die Chloroleukämie als heterogen anzusprechen ist. Neben extrem unreifen treten Chloroleukämien mit vorwiegend jugendlichen Granulozyten und nur sehr selten reifere auf. Zytologisch unterscheidet sich die Chloroleukämiezelle nicht von den entsprechenden Zellkategorien anderer Leukämien, lediglich durch den Besitz von Myeloperoxydase differiert sie. Vorherrschend finden sich im Blutbild die Paraformen von Myeloblasten, Promyelozyten oder Myelozyten. Auffallend ist der zwar in der Anzahl wechselnde, aber immer vorhandene Anteil von Lymphozyten. Daneben

treten auch einige Zellen der retikulären Reihe in Erscheinung. Der Absolutwert variiert, übersteigt aber nur in wenigen Fällen 100000. In der überwiegenden Mehrheit sind die Chloroleukämien generalisiert, vereinzelt kommen auch intrathorakal lokalisierte Formen vor. Die Chloroleukämie zeichnet sich zytochemisch durch eine ausgeprägte Peroxydase-Positivität aus, die sich teilweise bis zu den Paramyeloblasten erstreckt. Die alkalische Phosphatase-Aktivität ist nur bei den unreifsten Zellformen nachzuweisen, während die differenzierteren Chloroleukämien negativ sind. Saure Phosphatase ist negativ, Esterase bei allen Zellen schwach positiv.

Tabelle III

Aufspaltung in hämatologisch differente Leukämietypen. Filtrate von Chloroleukämien.

Leukämietyp	Fälle	Prozentsatz	Mäusestämme XVII	AB	CBA	Latenzzeit in Wochen
Myel. (differenz.) Leukämien	24	30,0	9	11	4	15,5
Unreife myeloische Leukämien	15	18,8	4	6	5	15,7
Chloro-Leukämien	11	13,8	7	3	1	20,1
Paramyeloblasten-Leukämien	18	22,5	6	8	4	15,1
Myelorisch-retikuläre Leukämien	9	11,2	5	3	1	13,2
Retikuläre Leukämien	2	2,5		1	1	23,5
Retikulär-lymphatische Leukämien	1	1,2		1		19
Lymphatische Leukämien	—					

Chloroleukämie Filtrate rufen nach Injektion in Mäusen die in der Tabelle III dargestellten Leukämieformen hervor. Aus der Tabelle ist ersichtlich, daß die Chloroleukämien eine merkliche Aufspaltungspotenz besitzen. Ca. 80 % der induzierten Leukämien gehören der myeloischen Reihe an, wobei sich differenzierte und unreife Leukämietypen ungefähr die Waage halten. Auffällig ist die gegenüber den unreifzelligen Leukämien zurücktretende Tendenz zur Ausbildung myelorisch-retikulärer Mischformen.

Paramyeloblasten-Leukämien (Abb. 4)

Der vorherrschende und bestimmende Zelltyp dieser Leukämieform ist der Paramyeloblast, der schon im Frühstadium der Leukämogenese im peripheren Blut auftritt. Im Hinblick auf die Zellbildung ist die Paramyeloblasten-Leukämie die einheitlichste Leukämieform. Differentialdiagnostisch läßt sie sich in den meisten Fällen durch die charakteristische Anordnung der Nukleolen, durch die Größe der Zellen und durch das Fehlen von Kernschatten sicher von der lymphatischen Leukämie abgrenzen. Die Paramyeloblasten-Leukämie tritt in vielen Fällen mit einem enorm hohen Absolutwert an Leukämiezellen in der Peripherie auf. Die Durchschnittswerte liegen zwischen 100–300000. Lokalisierte Formen sind in unserem Untersuchungsgut nicht vorgekommen. Zytochemisch ist vor allem die konstant starke Aktivität der alkalischen Phosphatase in den Paramyeloblasten zu erwähnen, die besonders in Gefrierschnitten leukämischer Infiltrate auffällig hervortritt. Die unspezifische Esterase ist konstant schwach positiv, während Peroxydase und saure Phosphatasen meist negativ sind.

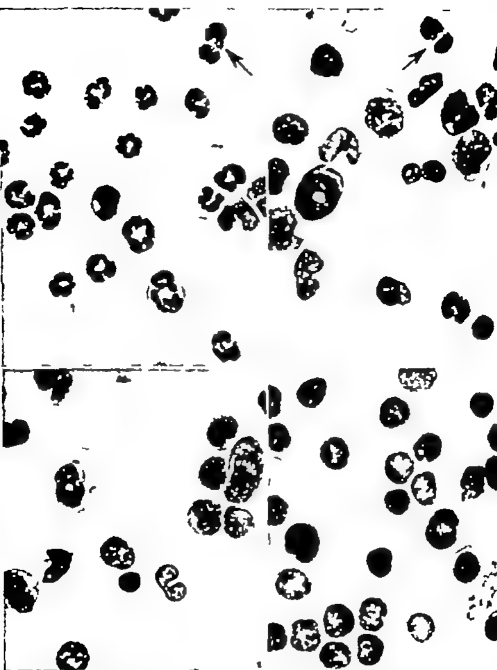


Abb. 1 Myeloische differenzierte Leukämie. Vorwiegend jugendliche ringkernige und reife neutrophile Granulozyten → Pseudo-Pelgerzelle. Giemsa. (800)
Abb. 2 Unreifzellige myeloische Leukämie. Vorherrschend Paraformen von Promyelozyten und Myelozyten → Pseudo-Pelgerzelle. Giemsa. (800

Nach Injektion von Paramyeloblasten Leukämiefiltraten treten die in der Tabelle IV angeführten Leukämietypen auf. Es ist zu ersehen daß die Paramyeloblasten Leukämie als Ausgangsmaterial eine deutliche Aufspaltungsneigung erkennen läßt, die bis zur Induktion lympathischer Leukämieformen führt. Sehr charakteristisch ist das Erscheinen vorwiegend unreifer Leukämiekategorien deren Anteil in diesen Versuchen über 50% beträgt. Sehr merklich treten die reifzelligen und Chloroleukamien zurück, während die retikulären Leukämien auffallend häufig sind

Tabelle IV

Aufspaltung in hämatologisch differente Leukämietypen Filtrate von Paramyeloblasten-Leukämien.

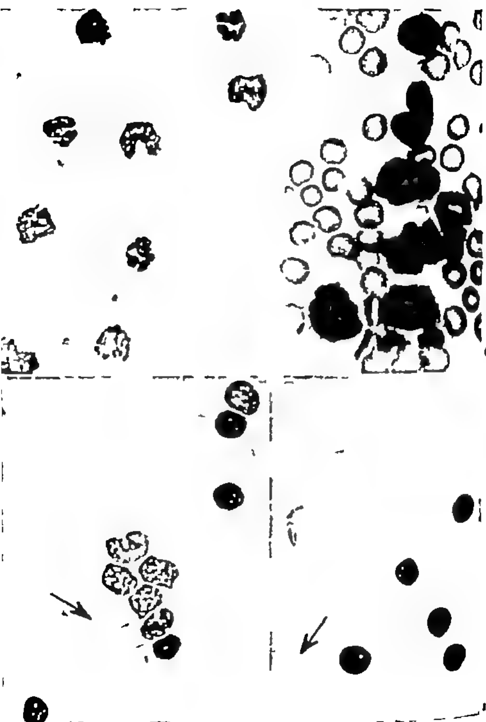
Leukämietyp	Fälle	Prozentsatz	Mäusestamm XVII	AB	CBA	Lebensdauer in Wochen
Myel. (differenz.) Leukämien	5	11,6	5			12,4
Unreife myeloische Leukämien	10	23,3	9	1		10 4
Chloro-Leukämien	1	2,3			1	22,0
Paramyeloblasten-Leukämien	13	30,2	10	2	1	13,0
Myeloisch-retikuläre Leukämien	9	20,9	7	2		13,2
Retikuläre Leukämien	3	7,0	1	1	1	14,6
Retikulär-lymph. Leukämien	1	2,3	1			11,0
Lymphatische Leukämien	1	2,3		1		13,0

Myeloisch-retikuläre Leukämien (Abb 5)

Die unter diesem Überbegriff zusammengefaßten Leukämien stellen echte Mischformen zwischen Leukämien der myeloischen Reihe und solchen dar deren Abkunft von RHS-Zellen angenommen werden muß Der Anteil der beiden Komponenten variiert in den meisten Fällen dominieren die leukämischen myeloischen Zellen. Der Reifegrad der beteiligten myeloischen und retikulären Zellen ist unterschiedlich. Man gewinnt jedoch den Eindruck, daß die reiferen Zellformen überwiegen. Die maligne Natur der Zellen beider Systeme läßt sich besonders eindrucksvoll an Infiltrationszentren demonstrieren, in denen sowohl myeloische als auch retikuläre Zellen ein invasives und destruierendes Wachstum zeigen. Die Anzahl der in der Peripherie vorhandenen Leukämiezellen ist im allgemeinen nicht sehr hoch und übertrifft 50000 nur ausnahmsweise. Zytochemisch läßt sich in den reiferen myeloischen Anteilen Peroxydase mit unterschiedlicher Aktivität nachweisen. Auch in den retikulären Zellen, die sich vorwiegend von Monozyten ableiten, kann Peroxydase in meist geringerer Menge beobachtet werden. Die alkalische Phosphatase ist negativ während saure Phosphatase in den retikulären Zellen deutlich in Erscheinung tritt. Auch die unspezifische Esterase zeigt bei letzteren eine erhöhte Aktivität.

Abb. 3. Chloroleukämie, überwiegend unreifzellige Paraformen von Myeloblasten Promyelozyten und Myelozyten. Mitte unten 1 Lymphozyt, oben links eine retikuläre Zelle. Giemsa. (800)

Abb 4 Paramyeloblasten-Leukämie Fast ausschließlich Paramyeloblasten, einige Lymphozyten. Giemsa. (× 800)



Die Aufspaltung der nach Injektion zellfreier Filtrate aus myeloisch-retikulären Leukämien auftretenden Formen ist in der Tabelle V dargestellt. Wie ersichtlich ist, ruft die myeloisch-retikuläre Mischleukämie ein Spektrum relativ reifzelliger Leukämien hervor, die mit ca. 60% den größten Anteil bilden. Andererseits besteht jedoch auch eine deutliche bis zu lymphatischen Leukämien reichende Aufspaltungstendenz. Der Anteil der Chloroleukämien erreicht fast die Werte, die bei unreifzelligen und Chloroleukämien als Ausgangsmaterialien ermittelt wurden.

Tabelle V

Aufspaltung in hämatologisch differente Leukämietypen. Filtrate von myeloisch-retikulären Leukämien.

Leukämietyp	Fälle	Prozentsatz	Mäusestamm XVII	AB	CBA	Lebensdauer in Wochen
Myel. (differenz.) Leukämien	24	36,4	14	10		13,6
Unreifz. myeloische Leukämien	9	13,6	2	7		14,3
Chloro-Leukämien	7	10,7	3	3	1	17,1
Paramyeloblasten-Leukämien	1	1,5		1		17
Myeloisch-retikuläre Leukämien	18	27,3	8	10		15,6
Retikuläre Leukämien	3	4,5		3		15,3
Retikulär-lymphatische Leukämien	1	1,5		1		18
Lymphatische Leukämien	3	4,5	2	1		13

Retikuläre Leukämien (Abb. 6)

Als retikuläre Leukämien fassen wir sämtliche Leukämietypen zusammen, die sich von Zellen des RHS ableiten, d. h. die Retikulumzell-, Histiozyten-, Monozyten- und Plasmozyten-Leukämien. Wir haben uns insofern zu dieser Maßnahme entschlossen, als die extrem unreifen retikulären Leukämien nicht mit Sicherheit auf ihre spezifischen Ausgangszellen zurückgeführt werden können. In dieser heterogenen Gruppe treten uns Leukämien der verschiedensten Reifegrade entgegen. Als weitgehend differenzierte Leukämieform ist die Monozytenleukämie anzusprechen, während die histiozytären und Retikulumzell-Leukämien meist sehr unreif sind. Plasmozyten-Leukämien sind in unserem Untersuchungsgut sehr selten. Je nach Abkunft der malignen Zellen ist ihre Anzahl in der Peripherie unterschiedlich. Während die differenzierteren Formen meist einen Absolutwert um 50000 aufweisen, finden sich bei den unreifen Typen

Abb. 5. Myeloisch-retikuläre Leukämie, vorwiegend reifzellig. Die retikuläre Komponente besteht aus leukämischen Monozyten. Giemsa. (× 800)

Abb. 6. Retikuläre Leukämie. Maligne Monozyten und Histiozyten. Giemsa. (× 1200)

Abb. 7. Lymphatisch-retikuläre Leukämie. In der Mitte oberhalb des Kernschattens (→) retikuläre Zellen. Die übrigen Zellen sind leukämische Lymphozyten unterschiedlicher Reifegrade. Giemsa. (× 800).

Abb. 8. Lymphatische Leukämie. Leukämische Lymphozyten, vorwiegend reifzellig. → Kernschatten. Giemsa. (× 800)

Werte bis zu 300000. Neben den generalisierten retikulären Leukämien konnten wir eine merkliche Anzahl von Retothelsarkomen beobachten, die wir dem Typ A von Dix (1) zuordnen. Differentialdiagnostisch unterscheiden sich die unreifen retikulären Leukämien, die im Erscheinungsbild den Paramyeloblasten-Leukämien sehr ähnlich sind, von diesen durch die andersartige Lokalisation der Nukleolen und vor allem durch das gänzliche Fehlen der alkalischen Phosphatase. Die bei solchen Fällen zu beobachtende deutlich erhöhte saure Phosphatase und unspezifische Esterase-Aktivität kann ebenfalls zur Diagnosestellung herangezogen werden. Bei den reiferen Monozyten- und zum Teil Histiozyten-Leukämien kann die Peroxydasereaktion positiv sein, die bei den erwähnten unreifen Formen immer negativ ist.

Tabelle 17

Aufspaltung in hämatologisch differente Leukämietypen. Filtrate von retikulären Leukämien.

Leukämietyp	Fälle	Prozentsatz	Mäusestamm XVII	Mäusestamm AB	Latenzzeit in Wochen
Myeloische (differenz.) Leukämien	7	22,6	2	5	13,2
unreife, myeloische Leukämien	3	~9 7	1	2	11,3
Chloro-Leukämien	3	~9 7	1	2	15,6
Paramyeloblasten-Leukämien	1	~3,2	1		13,0
Myeloisch-retikuläre Leukämien	9	29,0	5	4	11,9
Retikuläre Leukämien	8	25,8	3	5	12,1
Retikulär-lymph. Leukämien	—				
Lymphatische Leukämien	—				

Nach Applikation von Filtraten retikulärer Leukämien tritt die in Tabelle XVI dargestellte Aufspaltung ein. Aus der Tabelle geht hervor, daß über die Hälfte der induzierten Leukämien den rein retikulären oder den myeloisch retikulären Mischformen zuzuordnen ist. Während die reifzelligen myeloischen Leukämien ca. ein Viertel ausmachen, treten die unreifzelligen Formen zurück. Differenzierungen in lymphatischer Richtung fehlen ganz.

Retikulär-lymphatische Leukämien (Abb. 7)

Die Leukämien dieser Gruppe sind echte Mischformen zwischen malignen RHS- und lymphatischen Zellen. Im allgemeinen scheint die lymphatische Komponente den unreifen Anteil darzustellen, denn mitunter lassen sich im Blutausstrich nur wenige intakte lymphatische Zellen neben vielen sogenannten Kernschatten nachweisen. Die retikuläre Komponente besteht zum großen Teil aus differenzierteren Zellen, die der mono- oder histiozytären Reihe angehören dürften. Die retikulär-lymphatische Leukämie tritt gegenüber den bisher angeführten Leukämietypen seltener auf. Lediglich bei der Anwendung lymphatischen Ausgangsmaterials in zellfreien Übertragungsversuchen ist ihr Vorkommen häufiger. Die Anzahl der Leukämiezellen im peripheren Blut ist meist niedrig, die Durchschnittswerte liegen zwischen 20–40000. Zur einwandfreien Diagnostizierung dieser Leukämieform ist die Anwendung zytochemischer Methoden unerläßlich. Durch die deutliche stark alkalische Phosphatase-Aktivität der

maligen Lymphozyten läßt sich die Beteiligung der lymphatischen Komponente einwandfrei nachweisen und gestattet damit gleichzeitig eine sichere Abgrenzung gegenüber nichtmaligen lymphatischen Zellen. Die retikulären Zellen enthalten keine alkalische Phosphatase, zeigen jedoch eine erhöhte saure Phosphatase- und unspezifische Esterase-Aktivität.

Auf eine tabellarische Darstellung der Aufspaltungspotenz retikulär-lymphatischer Leukämien mußten wir infolge der geringen Anzahl verzichten.

Lymphatische Leukämien (Abb 8)

Die lymphatischen Leukämien unseres Untersuchungsgutes sind in ihrer Erscheinungsform einheitlich und differieren lediglich etwas im Reifungsgrad. Neben unreifen lymphoblasten-ähnlichen Zellen, die etwas größer als die ausgereiften Lymphozyten sind, kommen häufiger Leukämieformen mit den typischen kleinen Lymphozyten vor. Die malignen lymphatischen Zellen scheinen sich zu ungefähr gleichen Teilen von *Follikel- und Sinuslymphozyten abzuleiten, wie ihre Nukleolenanzahl und -lokalisation* erkennen läßt. Die Anzahl der malignen lymphatischen Zellen ist im Vergleich zu den myeloischen Leukämien auffallend gering. Nur in Ausnahmefällen wurden 50000 Zellen erreicht, der Durchschnitt liegt bei 30000. Auch einige Fälle von aleukämischen lymphatischen Leukosen konnten wir neben einer merklichen Anzahl von Lymphosarkomen diagnostizieren. Im Blutausstrich fallen die lymphatischen Leukämien durch die äußerst leichte Lädierbarkeit der malignen Zellen auf. Ausnahmslos finden sich in großer Anzahl die sogenannten «Kernschatten», die durch zerdrückte Zellkerne hervorgerufen werden. I einigen Fällen war die lymphatische Leukämie mit einer Begleit-Leukozytose vergesellschaftet, die Diagnosestellung wurde jedoch dadurch nicht wesentlich erschwert. Zytochemisch zeigen die malignen lymphatischen Zellen die stärkste alkalische Phosphatase-Aktivität aller Leukämiezellen. Die saure Phosphatase, unspezifische Esterase und die Peroxydase sind negativ

Tabelle VII

Aufspaltung in hämatologisch differente Leukämietypen. Filtrate von lymphatischen Leukämien.

Leukämietyp	Fälle	Prozentsatz	Mäusestamm XVII	AB	Lebensdauer in Wochen
Myeloische (differenz.) Leukämien	3	3,7	2	1	12,3
Unreife myeloische Leukämien	1	1,2	1		10,0
Chloro-Leukämien	1	1,2	1		14,0
Promyeloblasten-Leukämien	1	1,2		1	12,0
Myeloisch-retikuläre Leukämien	9	11 1	5	4	13,0
Retikuläre Leukämien	4	4,9	3	1	12,0
Retikulär-lymphatische Leukämien	14	17,3	9	5	12,2
Lymphatische Leukämien	48	59,3	25	[illegible]	12,2

In der Tabelle VII ist die Aufspaltungspotenz der lymphatischen Leukämien nach zellfreier Übertragung in Mäusen dargestellt. Sie ist primär nur geringgradig Lediglich ca. 7% myeloische und 16% myeloisch retikuläre bzw retikuläre Leukämien werden

Tabelle VIII
Aufspaltung einer myeloischen (differenz.) Leukämie in aufeinanderfolgenden zellfreien Passagen.

Leukämietyp	1. Passage Fälle	Prozentsatz	2. Passage Fälle	Prozentsatz	3. Passage Fälle	Prozentsatz
Myel. (differenz.) Leukämien	1	~11,1	7	31,8	10	30,3
Unreifz. myel. Leukämien	—		2	~9,1	3	9,1
Chloro-Leukämien	6	66,7	7	31,8	15	45,5
Paramyeloblasten-Leukämien	1	~11,1	3	13,6	1	3,0
Myeloisch-retikul. Leukämien	—		2	~9,1	4	12,1
Retikuläre Leukämien	1	~11,1	1	~4,5	—	
Retikulär-lymph. Leukämien	—		—		—	
Lymphatische Leukämien	—		—		—	

induziert. Annähernd 60% der entstandenen Leukämien bleiben lymphatisch und zeigen damit die höchste Konstanz aller untersuchten Leukämieformen

Um die Stabilität der einzelnen Leukämietypen in aufeinanderfolgenden zellfreien Passagierungen zu testen sind wir in unseren Versuchen von einer myeloisch differenzierten Leukämie und einer lymphatischen Leukämie ausgegangen. Die Ergebnisse sind in den Tabellen VIII und IX dargestellt. Aus der Tabelle VIII ist zu ersehen, daß der Charakter der Ausgangsleukämie in den nachfolgenden Passagen konstant beibehalten wird. Es dominieren in der 2 und 3 Passage die myeloisch-differenzierten und die Chloroleukämien, während die unreifen Leukämien höchstens einen Prozentsatz um 20 erreichen. Eine kleine Tendenz zur Differenzierung in retikulärer Richtung ist auch hier spürbar Im allgemeinen stimmen die Ergebnisse der einzelnen Passagen mit denen in der Tabelle I dargestellten gut überein. Die diesbezüglichen Verhältnisse bei der als Ausgang verwendeten lymphatischen Leukämie sind aus der Tabelle IX zu ersehen Auch hier läßt sich eine recht gute Stabilität der lymphatischen Leukämie ersehen. Ob die in der 3 Passage zu verzeichnende geringe Aufspaltungsneigung sich in weiteren Passagen verstärkt und zu einem eventuellen Rückschlag in die myeloische Differenzierungsrichtung führen könnte, müßte durch weitere Versuche geklärt werden.

Um eventuell vorliegende Unterschiede in der Aufspaltungstendenz der Leukämien bei unterschiedlichen Stämmen zu erfassen, haben wir in der Tabelle X Versuche zusammengestellt, in denen sowohl AB- als auch XVII Mäuse mit myeloisch differen-

Tabelle IX

Aufspaltung einer lymphatischen Leukämie in aufeinanderfolgenden zellfreien Passagen.

Leukämietyp	1. Passage Fälle	Prozentsatz	2. Passage Fälle	Prozentsatz	3. Passage Fälle	Prozentsatz
Myel. (differenz.) Leukämien	—		1	~3,9	2	~6,7
Unreife. myel. Leukämien	—		—		1	~3,3
Chloro-Leukämien	—		—		1	~3,3
Paramyeloblasten-Leukämien	1	~2,9	—		—	
Myeloisch-retikul. Leukämien	2	~5,8	2	~11,8	5	16,7
Retikuläre Leukämien	—		1	~5,9	3	10,0
Retikulär-lymph. Leukämien	6	17,6	3	17,6	5	16,7
Lymphatische Leukämien	25	73,5	10	58,8	13	43,3

Tabelle X

Aufspaltung in hämatologisch differente Leukämietypen nach Applikation von Filtraten myeloischer (diff.), Chloro- und lymphatischer Leukämien bei den Mäusestämmen AB und XVII.

Leukämietyp	Stamm Agouti Bahm (AB) Ausgangsfiltrat				Stamm XVII Ausgangsfiltrat			
	myel. (diff.) Leukämie	Chloro-Leukämie	lymph. Leukämie	Prozentsatz	myel. (diff.) Leukämie	Chloro-Leukämie	lymph. Leukämie	Prozentsatz
Myel. (differenz.) Leukämien	7	11	1	21,6	7	4	—	22,4
Unreife. myeloische Leukämien	3	8	—	12,3	8	4	—	20,4
Chloro-Leukämien	6	3	—	10,2	—	1	—	~2,1
Paramyeloblasten-Leukämien	3	6	1	11,4	2	6	—	16,3
Myeloisch-retikul. Leukämien	1	3	4	9,1	—	3	—	6,2
Retikuläre Leukämien	1	1	1	3,4	—	—	—	—
Retikulär-lymph. Leukämien	—	1	5	6,8	—	—	4	8,1
Lymphat. Leukämien	—	—	22	25,0	—	—	12	24,5

zierten, Chloro- oder lymphatischen Leukämiefiltraten behandelt wurden. Obwohl das Untersuchungsmaterial für eine solche Aussage relativ gering ist, dürfte sich doch mit hoher Wahrscheinlichkeit aus den Versuchen ergeben, daß die beiden Hauptdifferenzierungsrichtungen, nämlich die myeloische differenzierte Leukämie und andererseits die lymphatische Leukämie ein auf beiden genetisch differenten Mäusestämmen übereinstimmendes Verhalten hinsichtlich der Aufsplitterungspotenz zeigen.

Besprechung der Befunde

Bei der Diskussion unserer Befunde gehen wir zweckmäßigerweise von der Tatsache aus, daß bei Anwendung von Filtraten aus individuellen Leukämien eine Aufsplitterung in verschiedene Leukämieformen stattfindet, wie das auch in früheren Arbeiten von uns bereits beschrieben wurde (11). Diese Aufsplitterung ist, wenn auch in unterschiedlichem Maße und in etwas differenter Qualität, mit sämtlichen untersuchten Einzelfiltraten nachweisbar gewesen und außerdem auch bei allen benutzten Mäusestämmen.

Wie eingangs erwähnt wurde, sind Aufspaltungstendenzen auch bei anderen murinen virusinduzierten Leukämien in letzter Zeit bekanntgeworden. Gross (16) fand nach Rattenpassage des A-Virus das Auftreten verschiedener Leukämieformen. Auch nach Thymektomie bei C3H-Mäusen tritt eine Leukämiepalette auf, darunter auch Chloroleukämien (14, 15). Bei dem Mäusestamm BALB/c induziert das Grosssche Virus in einem relativ hohen Prozentsatz myeloische und unreife nichtlymphatische Leukämieformen (17). Auch das Friend-Virus ruft bei Ratten abweichende Leukämieformen hervor. Während in Mäusen nur retikuläre Leukämien erzeugt werden, kommt es bei Ratten zur Ausbildung lymphatischer Leukämien. Nach Rückpassagierung auf die Maus kommt es neben den erwähnten Formen auch zur Ausbildung von myeloischen Leukämien (18). Schließlich ist das primär retikulogene Rauscher-Virus noch zu erwähnen. Nach Untersuchungen von Siegel et al. (10) treten bei BALB-Mäusen auch Erythroblasten-Leukämien auf.

Bezüglich der Aufsplitterung in die verschiedenen Leukämieformen bestand bei den von uns benutzten Mäusestämmen sogar weitgehende Übereinstimmung, woraus sich ergibt, daß sie nicht erstrangig durch die genetische Konstitution bedingt sein kann (11). Zwischen dem Agnes-Bluhm-Stamm und Stamm XVII fand sich nur im Hinblick auf den Prozentsatz an Chloroleukämien, die bei letzterem nahezu völlig fehlten, eine größere Differenz, jedoch keineswegs in der Relation der Haupttypen zueinander, nämlich myeloische, retikuläre, lymphatische und gemischte Formen.

Das Gross-Virus scheint dagegen im Hinblick auf die Ausbildung unterschiedlicher Leukämietypen eine bestimmte Stammesspezifität zu besitzen. Während bei C3H-Mäusen ca. 1 % myeloische Leukämien induziert werden, treten nach Injektion desselben

Virusmaterials bei BALB/c Mäusen 20% myeloische Leukämien auf (17). Von FIORE DONATI UND CHIECO-BIANCHI (6) wird über den Einfluß der Wirtsreaktion auf die Entwicklung und den Typ der Leukämien berichtet, die durch unser Virus der myeloischen Leukämie der Maus induziert wurden. Sie finden bei allen untersuchten Stämmen, darunter auch beim Stamm XVII ein bevorzugtes Auftreten von lymphatischen Leukämien. Allerdings klassifizieren sie die entstandenen Leukämien nur in 3 Kategorien.

Im Gegensatz zur sekundären Bedeutung der genetischen Konstitution des Tiermaterials bei den eigenen Untersuchungen resultiert aus unseren vorausgehend beschriebenen Befunden, daß die hämatologische Form der zur Filtratherstellung benutzten Leukämie für den Grad und die Art der Aufsplitterung in die verschiedenen Leukämietypen von entscheidender Bedeutung ist. Wir wollen diese Verhältnisse, wie sie sich aus unseren Experimenten ableiten lassen, nochmals kurz darstellen. Filtrate aus reifen myeloischen Leukämien einerseits und typischen lymphatischen Leukämien andererseits wiesen die stärkste Konstanz der Wirkung im Hinblick auf die hämatologische Form der induzierten Leukämien auf, indem jeweils weit überwiegend die identische Leukämieform bei gleichzeitig relativ geringer Aufsplitterung reproduziert wurde. Diese relative Konstanz der hämatologischen Form bleibt auch nach mehreren zellfreien Passagen erhalten, wenn auch speziell bei den lymphatischen Leukämien eine Zunahme der Aufsplitterungstendenz im Laufe der zellfreien Generationen angedeutet wurde. Andererseits ist mit Filtraten unreifer myeloischer Leukosen und der Paramyeloblasten-Leukämien stets eine starke Aufspaltung in die verschiedensten Differenzierungsformen einschließlich Mischformen zu verzeichnen, d. h. also, die Tendenz zur qualitativen und quantitativen hämatologischen Aufsplitterung ist bei Anwendung von Filtraten aus unreifzelligen Leukosen beträchtlich größer als bei den verschiedenen extrem differenzierten reifen Typen. Dabei resultieren im Zuge der Aufsplitterung stets neben unreifen Formen beträchtliche Prozentsätze an reifen Leukämien sowohl der myeloischen als auch lymphatischen Reihe, außerdem noch retikuläre Formen sowie Mischformen aus letzterem Typ mit myeloischen Elementen einerseits und lymphatischen andererseits. Es ist bemerkenswert, daß Mischformen nur beim retikulären Typ angetroffen wurden, niemals jedoch die Kombination lymphatischer mit myeloischer Komponente nachweisbar war. Es ist weiterhin hervorzu-

Besprechung der Befunde

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Es muß allerdings zunächst noch völlig offenbleiben, in welcher Weise die lange Latenzzeit kausal mit der Ausbildung von Chloroleukämien verbunden ist. Eine an sich abgeschwächte Virusvirulenz und entsprechend abgeänderte Wirkungsweise des Virus auf die Zelle bei der malignen Entartung (Reife- und Differenzierungsgrad der Zelle, Fermentausstattung derselben, etc.) käme möglicherweise ebenso in Betracht wie bestimmte besondere biologische Bedingungen für das gesamte blutbildende System, die im späteren Lebensalter der Tiere in höherem Maße gegeben sind als in den ersten Lebenswochen. Es ist also eventuell daran zu denken, daß das ältere Tier auf das Virus aus Gründen einer besonderen Reaktionsweise seiner myeloischen Zellen bevorzugt mit Chloroleukämie, also mit der Ausbildung einer spezifischen Fermentopathie respekt. (Verdoperoxydase), während im jüngeren Alter in erster Linie myeloische Leukämien ohne Verdoperoxydase auftreten.

Für die Abhängigkeit bestimmter Leukämieformen vom Alter der Tiere spricht auch die Tatsache, daß Paramyeloblasten- und andere unreife Leukämien im höheren Alter der Tiere selten sind, während sie bei jungen Tieren oft die häufigsten Leukämieformen darstellen. Für die Chloroleukämien trifft das Umgekehrte zu.

Schließlich noch einige Bemerkungen zur Frage des Mechanismus der Aufsplitterung der Leukämieformen bei der zellfreien Übertragung. Drei verschiedene Möglichkeiten können, von seiten des Virusmaterials aus betrachtet, in Erwägung gezogen werden:

1. Die Aufsplitterung ist dadurch bedingt, daß das Virusmaterial aus einem Gemisch mehrerer verschiedener fixierter Virustypen besteht, von denen jeder Typ jeweils nur eine ganz bestimmte Leukämieform auszulösen vermag.
2. Es gibt nur einen Virustyp, der entsprechend den jeweiligen Bedingungen oder eventuell auch rein statistisch verschiedene Stammzelltypen oder Jugendformen des hämatopoetischen Systems zu infizieren und maligne zu transformieren vermag.
3. Die Möglichkeit, daß sich das Virus selber spontan oder entsprechend den Bedingungen, die es im Wirt vorfindet, zu wandeln vermag, wobei immer wieder eine Aufsplitterung in Modifikationen mit differentem Histotropismus stattfindet.

Weitgehend ausschließen können wir von diesen drei Möglichkeiten auf Grund unserer Ergebnisse den zweiten Fall und zwar auf Grund der Tatsache, daß der hämatologische Typ der zur Filtratbereitung benutzten Leukämie die hämatologische Form der induzierten Leukämien wesentlich mitbestimmt. Dieses gilt, wie wir bereits erwähnt haben, vor allem für die ausgereiften myeloischen Leukämien und lymphatischen Leukosen. Im Fall eines
fest determinierten Virustyps würde die Herkunft des Filtrats
Rolle spielen, sondern vor allem die Bedingungen der V

tiere von entscheidender Bedeutung sein. Auch die erste der genannten Möglichkeiten scheint uns weniger wahrscheinlich zu sein. Hingegen würde die Abhängigkeit der Leukämieformen von der hämatologischen Form der zur Filtratvorbereitung benutzten Leukose zwanglos mit der dritten Möglichkeit in Einklang stehen. Dabei ist möglicherweise der Umstand, daß das Virus in Zellen eines ganz bestimmten hämatologischen Typs reproduziert wird, die unmittelbare Ursache für seinen jeweilig dominierenden spezifischen Histotropismus. Es wäre denkbar daß dies damit zusammenhängt, daß bei der Virusreproduktion an der Zelloberfläche oder an den zytoplasmatischen Membranen jedem einzelnen Viruspartikel Bestandteile (Antigene, Rezeptoren) der jeweiligen Mutterzelle mitgegeben werden, durch die die spezifische Affinität zum gleichen Zelltyp hergestellt wird In diesem Sinne spricht eventuell auch der Umstand, daß die Konstanz dieser Histotropie gerade bei dem Virusmaterial aus Leukämien höchster Differenzierungs- und Reifungsgrade (ausgereifte myeloische und lymphatische Leukosen) am größten ist und andererseits die unreifen myeloischen Leukosen, in denen der spezifische Zellcharakter am schwächsten ausgeprägt ist, ein Virus reproduzieren, das zu der qualitativ und quantitativ stärksten Aufsplitterung in hämatologisch verschiedene Leukämieformen führt.

Zusammenfassung

Die durch das mehrfach zellfrei passagierte Virus der myeloischen Leukämie der Maus induzierten Leukämien zeigen spontan eine Aufsplitterung in verschiedene hämatologische Formen, die von der Form der zur Virusisolierung benutzten Ausgangsleukose abhängig ist. Genetische Bedingungen des Tiermaterials ließen nur einen geringen Einfluß erkennen. Die geringste Aufsplitterungstendenz zeigten reifzellige myeloische und lymphatische Leukosen. Virusmaterial aus unreifzelligen myeloischen Leukosen ergab die stärkste hämatologische Aufsplitterung, wobei neben unreifen myeloischen Formen vermehrt retikuläre Leukämien und deren Mischformen mit myeloischen Komponenten sowie einige lymphatische Leukämien entstanden. Retikuläre Leukosen hingegen zeigten eine Aufsplitterung in myeloischer Richtung.

Die möglichen Ursachen und Mechanismen dieser hämatologischen Aufsplitterung und die Abnahme der Chloroleukämien im Vergleich zu früheren Versuchen werden diskutiert. Es wird auf die T tsache hingewiesen, daß der Leukämietyp von der hämatologischen Form der zur Filtratbereitung benutzten Ausgangsleukämie wesentlich mitbestimmt wird.

Summary

Leukaemia induced by the mouse myeloid leukaemia virus after several cell-free filtrations spontaneously takes on one of several forms, depending on the nature of the leukaemia used for the initial isolation of the virus. Mature myeloid and lymphatic leukaemia showed the least tendency to diversification, while virus material from

immature myeloid leukaemia showed the greatest. In this case in addition to immature myeloid forms, there were increasing numbers of reticular and mixed forms with myeloid components and few lymphatic forms. Reticular leukaemia, on the other hand, showed similar tendency to produce myeloid forms.

The authors discuss the possible causes and mechanisms of haematological diversification, and the reduction of chloroleukaemia in comparison with previous studies. It is emphasized that the type of leukaemia produced is very largely determined by the haematological form of the initial leukaemia used to prepare the filtrate.

Résumé

Le virus murin de la leucémie myéloïde provoque, après voir subit plusieurs filtrations sans cellule, des leucémies qui prennent spontanément différentes formes hématologiques, suivant l'espèce de la leucose employée pour l'isolement initial du virus. Les qualités génétiques de différentes souches d'animaux n'ont guère d'influence sur ces variations. Les virus isolés de leucoses myéloïdes immatures provoquent, à comparer à ceux de leucoses myéloïdes et lymphatiques, une diversité beaucoup plus grande de formes hématologiques des formes myéloïdes immatures, des formes réticulaires, des formes mixtes avec composante myéloïde et quelques leucémies lymphatiques. Les leucoses réticulaires d'autre part, montrèrent une tendence à produire des formes myéloïdes. Les causes possibles et les méchanismes de ces changements hématologiques ainsi que la diminution des chloroleucémies par rapport à des expériences antérieures sont discutés. Il est insisté sur le fait que le type de leucémie est déterminé en grande partie par la forme hématologique de la leucémie initiale employée pour la préparation du filtrat.

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The Occurrence of an Abnormal Cell in Murine Virus-Induced Leukemia*

By Jeanne C. Hopkins and Benjamin V. Siegel

The presence of an abnormal cell in peripheral blood in a number of disease states has been variously described and classified in humans by such investigators as Sprunt (1), Downey (2) and Litwins (3) and in mice by Frank (4) and Metcalf (5). A number of names, including virocyte (3, 12), adrenal insufficiency lymphocyte (4), plasmacytoid cell (6), virolymphocyte (3, 11), Friend cell (5) and undifferentiated cell, have been devised to identify it. In recent studies in our laboratory, mice inoculated with a murine leukemogenic (Rauscher) virus have been observed to manifest a similar abnormal cell phenomenon during the course of leukemia development. The present report purports to describe the experimental induction and development of this unusual cell type.

Material and Methods

The virus (7) employed in these experiments was received from Dr. Frank J. Rauscher of the National Cancer Institute and has been through five serial mouse passages in this laboratory. Virus-induced leukemic spleens from passages 2 to 5 served as source material for the preparation of centrifuged concentrates of the leukemia agent. These were stored as 30% suspensions in sealed ampoules at —70 °C and prior to use were diluted to 10[illegible] with Hanks' balanced salt solution containing 100 units of penicillin and 100 μg of streptomycin per ml. Two-tenths ml of the diluted virus preparation was inoculated intraperitoneally into 5 to 6 week old female BALB/C/Jax mice obtained from Jackson Memorial Laboratory. At various times during the course of infection, ranging from 2 weeks to 35 weeks, differential counts of 100 to 300 nucleated cells were carried out on peripheral blood obtained by snipping the tail. Mice were sacrificed at these times and imprints made of the spleens and livers. A total of 105 mice were examined in this way. Uninoculated control mice were similarly followed. The blood smears were stained by the Wright-Giemsa procedure (8) and spleen and

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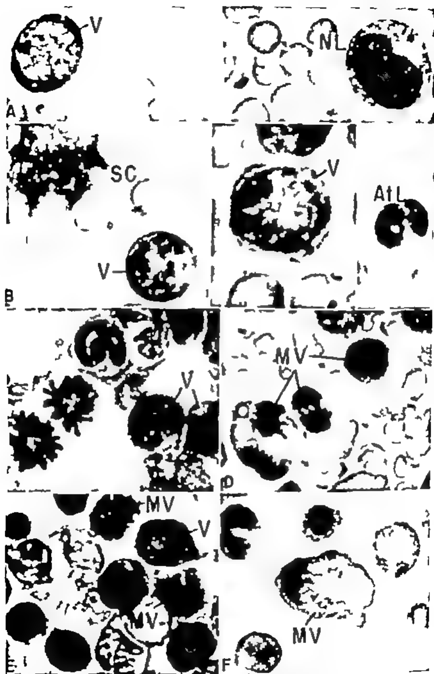


Fig. 1 Mouse peripheral blood during leukemogenic virus infection. Legend V (V-cell), NL (normal lymphocyte) SC (smudge cell) AtL (atypical lymphocyte) MV (mitotic V-cell) Wright-Giemsa X 1300. — A. An average-size V-cell is seen in early ovoid form in the same field with normal lymphocyte. — B. Two V-cells are seen with

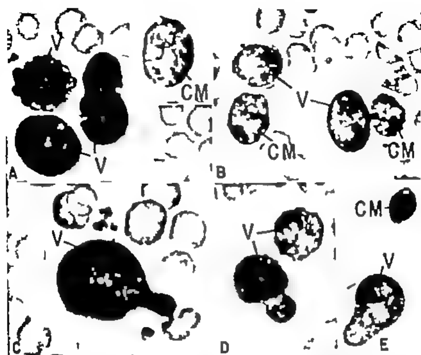


Fig. 2. Leukemic mouse blood showing V-cells (V) and cytoplasmic masses (CM). Wright's-Giemsa X 1500. — A. V-cell forms insert shows accompanying cytoplasmic mass which was seen in the same field. — B. Small V-cells with associated large cytoplasmic masses, one of which is noted to contain basophilic inclusion bodies. — C. A budding V-cell is prominent in this field. — D. Budding and ovoid V-cells containing coarse mitotic chromatin. — E. Budding V-cell with accompanying small cytoplasmic mass.

liver imprints were stained by the May-Grünwald-Giemsa method (9). In further experiment the blood picture changes in 41 infected and 8 control mice were followed in each individual animal by weekly orbital bleedings (10) over period of 19 weeks. Spleen and liver imprints of leukemic animals were made at the time of, or shortly after death.

Results

The nomenclature of V-cell was adopted here for the abnormal cell to indicate the induction of its formation, at least in the present instance, in a disease state of viral etiology The V-cell

smudge cell and an atypical lymphocyte. — C. I this field V-cells are observed along with granulocytes and mitotic cell. — D. Two of three V-cells in mitosis. Note the definite vacuolation and dark foamy cytoplasm of these mitotic cells. — E. V-cells, two of which are mitotic, are noted in the same field with granulocyte, normal lymphocytes and immature erythrocytes. — F. Early red blood cell forms with mitotic V-cell simulating cell structures found in megaloblastic erythropoiesis.

(fig. 1 A) varied in size from 7 to 20 μ in diameter was composed of scant to moderate amount of cytoplasm, appearing foamy deeply basophilic, sometimes vacuolated and with or without granules and basophilic inclusions. The pseudopodia-like projections of the cytoplasm of the X-cell recall the description by ACKERMANN AND BELLIOS (13) of the more mature leukemic cells with increased ameboid activity and irregular cytoplasmic contour (fig. 2 A E) The nucleus was basophilic, usually oval or indented, and at times appeared binucleated or lobulated. Nucleoli were occasionally apparent and the nucleoplasm at times presented a vesicular appearance with little organization of the scant, irregular-sized chromatin. Some nuclei appeared almost homogeneous with the cytoplasm, the thin nuclear wall providing the only clear delineation. Areas were present where portions of the nuclear wall and the cytoplasm merged and were difficult to distinguish as distinct entities. In other cells the nucleus was eccentrically located, with, occasionally a linear array of coarse chromatin across the diameter of the nucleus as a prominent feature. Mitotic figures occurred infrequently and were noted mainly in advanced stages of the disease (fig 1 C-F)

Ovoid or spherical non nucleated bodies ressembling X-cell cytoplasm and varying in size from 6 to 15 μ in diameter appeared concurrently in the blood of infected mice (fig. 2 A, B, E) These cytoplasmic masses were observed on occasion to be budding off from the X-cell, suggesting their possible origin in this manner (fig 2 A E)

A comparable cell form has thus far been reported in only one other mouse blood dyscrasia of viral origin, that by METCALF et al (5) who assigned the name FRIEND cell to this cell form occurring in RF and Swiss mice infected with FRIEND virus. Cytoplasmic masses accompanying the FRIEND cell were also described by these authors.

X-cells began to appear in the peripheral blood of infected mice at about 14 days following viral inoculation. They were present to the extent of 2 to 6 % in individual mice and varied little with duration of infection until the animals became demonstrably leukemic when their number increased to 8 to 10 %. METCALF et al. (5) noted a marked increase in the number of FRIEND cells to 10 to 20 % of the nucleated cells with development of the FRIEND disease.

In the earlier stage, from 4—10 weeks, of the Rauscher virus-induced leukemia, the V-cell was of a size similar to that of the average normal lymphocyte and contained a moderate amount of cytoplasm with an oval nucleus. With progression of the disease, there occured increasing enlargement of the cell along with cytoplasmic and nuclear variations. The latter included enhanced basophilia, vacuolation, and budding of the cytoplasm with appearance in some V-cells of eccentrically located nuclei; the nucleoplasm was of a *vesicular character with variations in chromatin* aggregation and arrangement and occasional mitotic figures (fig. 1 C-F; fig. 2 A-E). The accompanying cytoplasmic bodies simultaneously manifested changes similar to those noted in V-cell cytoplasm. V-cells were also observed in spleen and liver imprints during the course of the infection and became more prevalent in advanced stages of the disease along with an increase in normal lymphs and immature erythrocytic forms.

The appearance of the V-cell in mice followed on an individual basis by weekly orbital bleedings was similarly established at 14 days after virus inoculation. Immature and abnormal cells of the erythrocytic series appeared by 5 weeks postinoculation, persisting and increasing to 30% of the total nucleated count by 8 weeks with a few atypical lymphocytes present. The peripheral blood changes here duplicated closely the results of a previous study in which erythroleukemia was a prominent feature (14).

A review of differential nucleated cell counts from the aforementioned study (14) of peripheral blood taken up to 64 days following Rauscher virus inoculation similarly revealed the presence of V-cells. Unlike in the present experiments, however, these cells and accompanying cytoplasmic masses were seen relatively infrequently. The preparation in the present series of thin smears and the employment of the Wrights-Giemsa in place of the Wrights stain appeared to enhance the differentiation of nuclear structure making feasible a finer characterization of blood cells.

In consideration of the possible role of a sex factor, 10 uninoculated male BALB/C/jax mice were followed by weekly orbital bleedings. Over a period of 4 months there were no indications of V-cells or cytoplasmic masses in any of the blood smears made on these animals.

Discussion

Because of the structural variation in the arrangement and character of the nuclear chromatin, affinity of the vesicular cytoplasm for basic stain, mobility of the cytoplasmic wall, and occasional clasmatosis, the V-cell has not been cytogenetically classified by the authors. Its presence in the differential, accompanied by numerous normal and atypical lymphocytes and smudge cells (fig 1 A, B) might suggest a lymphocytic character The week prior to death the per cent of smudge cells increased markedly and the number of lymphocytes showed a decrease, the per cent of abnormal cells increasing and becoming more bizarre with the appearance at times, of mitotic forms (fig 1 C-F) There was increased polychromatophilia, HOWELL JOLLY bodies, basophilic stippling anisocytosis and poikilocytosis of erythrocytes along with the appearance of metarubricytes (10 to 30) and rubricytes (2 to 5) At this stage it was difficult to identify the larger abnormal cells with either lymphocytes or cells of the erythrocytic series. While the appearance of peripheral blood smears was generally erythroleukemic in some mice the blood picture simulated the megaloblastic erythropoiesis of human marrow in severe pernicious anemia (fig 1 F)

Although this abnormal cell has been reported in a number of diseases of viral etiology its occurrence with extracellular cytoplasmic bodies has not been described in these same instances One exception is the reference by METCALF (5) to a cytoplasmic mass in peripheral blood smears of Friend virus-induced leukemic mice Extracellular cytoplasmic structures, as already noted, have likewise been observed in the present experiments with mice infected with Rauscher virus.

The V-cell described here is reminiscent of cells reported in a number of human diseases of viral etiology (2 3 11) Such cells have also been described in situations of a non-infectious nature FRANK AND DOUGHERTY (4) observed the presence of a similar type cell in the peripheral blood of adrenalectomized mice and TROWELL (5) has described its occurrence in humans after chronic exposure to very low doses of ionizing radiation.

In view of the induction of this cell form in a variety of situations of heterogeneous causation, it is difficult to ascribe any clear cut specificity to the etiology or cytogenesis of this unique cell

form. An interference in the normal maturation of stem cells (15) into mature lymphocytes might explain the formation of this cell which presents features observed in abnormal erythrocytic and lymphocytic development. It would seem that the direct introduction of a virus or its latent activation by some nonspecific stress might be responsible for the induction of a host response characterized by production of an abnormal cellular form, such as the depicted V cell. The occurrence of these abnormal cells with cytoplasmic bodies in the peripheral blood in mouse leukemias induced by the Rauscher and Friend agents might further suggest a pathognomonic attribute of this cell in virus-induced leukemias.

Summary

An abnormal cell, designated as V-cell, has been observed in the peripheral blood of mice beginning about 14 days after inoculation of murine leukemogenic (Rauscher) virus. These V-cells are usually accompanied by characteristic extracellular cytoplasmic masses, which have also been described in mouse leukemia induced by Friend virus. Developmental forms of the cell are described and its possible viral etiology discussed.

Résumé

Une cellule anormale désignée sous le nom de cellule V a été observée dans le sang périphérique de la souris, à partir du quatorzième jour après l'inoculation d'un virus leucémigène murin (Rauscher). Ces cellules V sont habituellement accompagnées de masses plasmatiques extracellulaires qui ont aussi été décrites dans la leucémie de la souris, provoquée par le virus de Friend. Les formes évoluées de cette cellule sont décrites et leur origine virale est discutée.

Zusammenfassung

Im peripheren Blut von Mäusen wurde etwa 14 Tage nach Inokulation eines leukämogenen Mäusevirus (Rauscher) eine abnorme Zelle beobachtet, die als V-Zelle bezeichnet wird. Diese V-Zellen sind gewöhnlich von charakteristischen extrazellulären Massen begleitet, die auch bei einer durch das Friend-Virus hervorgerufenen Mäuseleukämie beschrieben wurden. Die Entwicklungsformen der Zelle werden dargestellt. Die Möglichkeit ihrer Entstehung durch das Virus wird diskutiert.

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Protective Effects of Erythropoietin Against Myleran Induced Hematodepression*

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The similarities between the peripheral hematopoietic effects of x-radiation and those of Myleran and Leukeran were described by Elson (1). When Myleran was administered to rats in a relatively small dose (15 mg/kg) a gradual fall in circulating neutrophils to 10% of the normal value occurred twelve days post treatment, while larger doses caused an additional decrease in red blood cells and lymphocytes. For this reason, Myleran was said to mimic the myeloid effects of x-radiation (400—600 r). On the other hand, it was found that Leukeran produced a selective and immediate decrease in lymphoid elements, thus mimicking the lymphoid effects of radiation. The complementary effects of simultaneous administration of Myleran and Leukeran were characteristic of both myeloid and lymphoid effects of radiation.

Elson (2) also found that the inhibition of hemopoiesis in the marrow of Myleran treated rats had been preceded by a recovery phase characterized primarily by an erythrocytosis. It was suggested that the latter abortive regeneration was the result of an inhibitory effect on the stem cell compartment, which consequently produced a diminished supply of erythroblasts and an inability to continue erythroid regeneration. Further indication that Myleran inhibits precursor cells rather than the maturation of their differentiated offspring is the fact, that the drug also inhibits proliferation of precursors of other cell types, e.g., spermatogonia (3).

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By applying a model of bone marrow stem cell kinetics proposed by LAJTHA (4 5) to data on Myleran action, GALTON (6, 7) was able to correlate the peripheral hemopoietic status with that of the marrow According to ELSON Myleran exerts its action most noticeably on cells which have the longest mitotic cycles, i.e., cells which do not have the capacity for quick regeneration. However the frequency of division of marrow cells estimated from radioisotopic studies is greatest for erythroblasts, and less for neutrophils, megakaryocytes and lymphocytes in order of decreasing frequency (4) The apparent discrepancy between the comparative decreases in peripheral values and those which would be anticipated according to established division rates for marrow cells is probably the result of different life spans of the various cell lines rather than a difference in sensitivity to the drug If this explanation is accepted, it is a good possibility that Myleran does affect the stem cell population in a non-specific manner If it is further assumed that precursors of erythroblasts divide less rapidly than do erythroblasts, then it would be expected that they would be the first to succumb to the increased intermitotic interval attributed to the action of Myleran. Thus, as first suggested by ELSON erythroblasts already present at the time of treatment are able to mature, but eventually their numbers become diminished because of a decreased supply of precursors.

The question as to how Myleran affects stem cells is not yet answered. It may inhibit DNA synthesis and proliferation, or RNA synthesis and differentiation, or both. There is substantial evidence that Myleran inactivates sulfhydryl groups and consequently interferes with purine synthesis (8, 9)

One of the more fruitful ways of finding a solution to this question might be an analysis of the effects of various protective agents against Myleran. TALBOT AND ELSON (10) have shown that protection against a dimethyl homologue of Myleran can be accomplished with isologous marrow transfusions. It is well known that this treatment is also effective against lethal doses of radiation (which bears many similarities to Myleran in its effect on hemopoietic tissues) Furthermore, erythropoietin has been used successfully to counteract the effects of radiation.

With this information at hand, it seemed that it would be profitable to attempt protection against the effects of Myleran with erythropoietin. If LAJTHA s model is used as a tool for studying the

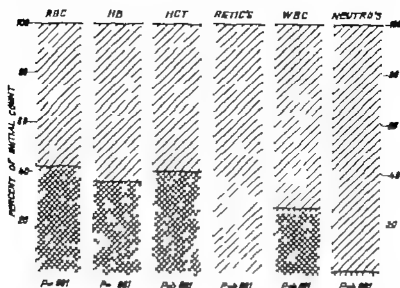


Fig. 1 Hematodepressant effects of chronic doses of Myleran. (P values indicate deviations from the initial)

effects of erythropoietin on Myleran treated animals, it might be possible to demonstrate the site of erythropoietin action as well as the mechanism of Myleran action.

Since this laboratory is also interested in elucidating the role of the spleen in hemopoiesis, all experiments were duplicated in splenectomized animals. Rather than the single, oral dose of Myleran used by ELSON we administered low doses for the duration of the experiment so that the hemopoietic status of the animals would remain consistent, and any effect of erythropoietin would not be minimized by a normal regenerative process. It is interesting to note that animals treated in this way become almost 100 % lymphocytic, thus providing a useful tool for studying the possible role of lymphocytes in erythropoiesis.

Materials and Methods

Adult female rats of modified Long-Evans strain (160—200 gm) were used in all experiments. Standard laboratory rations and water were supplied ad libitum.

Erythrocyte and leukocyte quantitations were made from tail blood in duplicate and required to agree within 4% and 10% respectively. Wright-stained smears were used for differential counts. Reticulocyte percents were estimated per thousand cells from smears of peripheral blood stained with new-methylene blue. Hemoglobin concentrations were determined by the acid-hematin method with photo-electric color-

meter Duplicate microhematocrit determinations (± 1.0 mm) were made on heparinized tail blood, spun at 12,000 rpm for 10 minutes. Pre-experimental hematologic determinations were considered as control values for each animal.

At the termination of each experiment, all rats were anesthetized lightly with ether and exsanguinated by cardiac puncture. Bone marrow differentials were made on smears stained with May-Grunwald or treated with the peroxidase method of RALPH.

Splenectomies were performed immediately prior to treatment under light ether anesthesia.

Myleran was suspended with tragacanth in distilled water and administered in oral doses of 1 mg per rat daily for 18 days. Three cobalt units of sheep plasma erythropoietin (Armour) were given daily for the duration of the experiment. Imprints of thymus, and lymph nodes (and spleen when present) were made at autopsy

Results

Percent decreases in the various peripheral hematologic parameters studied after eighteen days of treatment with Myleran are illustrated in fig 1 The 75% drop in total leukocyte numbers which is partially due to a 100% depression in neutrophils is characteristic of high doses of this drug A concommitant reduction in erythrocyte numbers, very significant decreases in hemoglobin and hematocrit values, and complete absence of reticulocytes is a manifestation of pronounced bone marrow inactivity Analysis of the bone marrow at this time reveals a marked aplasia characterized

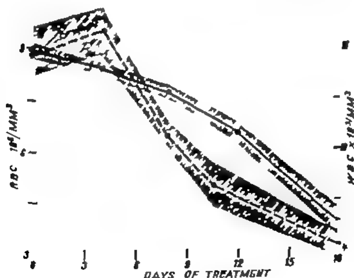


Fig 2. Rate of erythrocyte and leucocyte decreases following Myleran administration. (Shaded areas indicate standard deviations.)

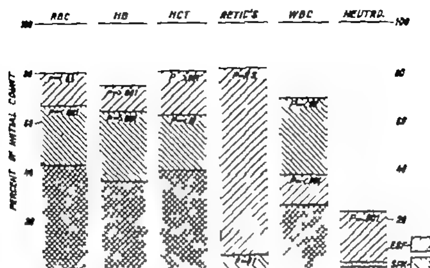


Fig 2. Protective effects of ESF against hematodepression of Myleran. (P values indicate deviations from the intact.)

by the presence of mast cells, extensive lymphocytic invasion and large numbers of histiocytic elements (fig 5) When compared to the normal nucleated red cell population (20—25%) the marrows of Myleran treated animals disclosed significantly lower values (3 0—8.0%)

The rate at which erythrocyte and leukocyte decreases occurred during the experimental period may be observed in fig 2. Subsequent to a brief initial leukocytosis, there is a progressive fall in all peripheral elements. It is interesting to note the absence of the abortive regeneration described by Elson (1) when lower single doses of the drug were administered

Autopsy examination at 18 days revealed degeneration of the spleen, lymph nodes, thymus and intestine. Histological studies of the kidney showed marked tubular degeneration. The progressive weight reductions observed during the experimental period were attributed to the generalized toxic effects of Myleran.

Fig. 3 compares the response of splenectomized animals and intact animals to similar doses of Myleran. It is evident that while significant decreases occur in all parameters, they are less severe in the splenectomized series. The protection afforded white blood cells

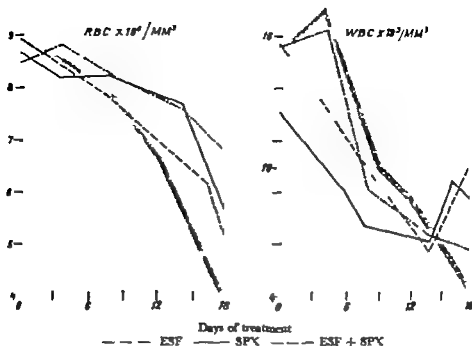
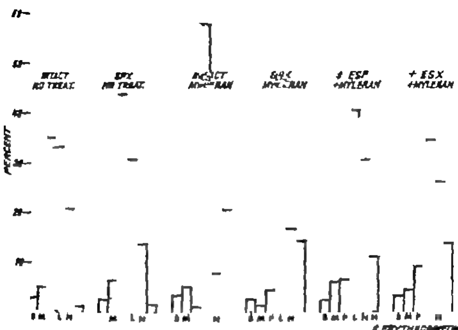


Fig 4 Comparison of protective effects of ESF and splenectomy

is confined to lymphocytic elements as reflected by the total leukocyte and differential counts.

Marrow aplasia and the appearance of histiocytes and reticulum cells occurred to a much lesser degree in the splenectomized animals than in intact Myleran treated controls. Consistent with this, is the significantly higher value of nucleated erythroid cells (17 0%) in these marrows. Lymph nodes, thymus and intestine exhibit a marked decrease in degree of atrophy when compared to non-splenectomized animals (fig 4)

The protective effects of erythropoietin and splenectomy against Myleran are compared in fig 3 and 4 Erythropoietin afforded greater protection in all red cell parameters than did splenectomy although values for both groups are significantly higher than those of intact animals receiving only Myleran. Splenectomy was more protective than erythropoietin against leukopenia. While protection offered by the latter was predominantly of lymphocytes, neutrophils were least depressed in the presence of erythropoietin. The greater protective capacity of erythropoietin is



B = Blasts M = Myelocytes, Pro- Meta- P = Polynucleated neutrophils L = Lymphocytes N = Nucleated red cells H = Histiocytes.

Fig. 5. Bone marrow at 18 days of treatment.

reflected by marrow nucleated red cell percentages which were for erythropoietin-treated intact animals 31.0 %, in contrast to 17.0 % for splenectomized animals receiving no erythropoietin. When splenectomized animals were treated with erythropoietin, the marrow nucleated red blood cell percent was 26.6 %. As indicated in fig. 4 the peripheral protection afforded by erythropoietin was minimized when the animals were also splenectomized. Autopsy examination indicated that all animals receiving erythropoietin suffered less from the toxic effects of Myleran than animals receiving the drug alone.

Discussion

The results indicate that erythropoietin is significantly protective against the peripheral and bone marrow hematological effects and toxic manifestations induced by Myleran. It was also shown that splenectomy is protective against Myleran, but to a lesser

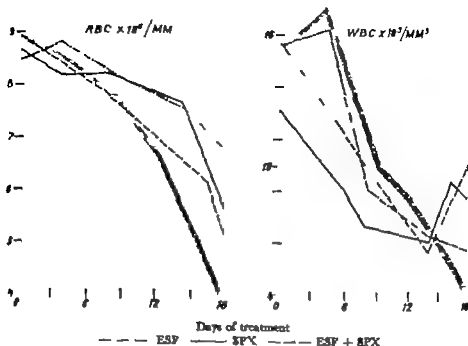


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hemopoietic inhibitor (12, 13) However the fact that splenectomy must be performed immediately prior to or immediately after treatment with Myleran and the failure of these animals to respond well to erythropoietin indicates that the protection afforded may be due to a more basic mechanism than those mentioned above. It has been shown that splenic artery exclusion prevents the toxic effects of alkylating agents from occurring in that organ, but does not improve survival percentages (14) Splenectomy prior to drug administration or transfusions of splenic homogenates also did not improve survival time. It seems then, that the protection afforded by the spleen is chiefly due to its removal at a critical time (i.e., at the commencement of drug treatment) Again, this phenomenon can be explained in light of the stem cell model. If a relatively large fraction of stem cells and differentiated cells are removed from the hemopoietic system while it is still capable of a response, compensation of the stem cell compartment can be anticipated. It is possible that this activity is sufficient to overcome the mitotic delay caused by Myleran at least temporarily

Protection against hematodepressant effects of alkylating agents is also afforded by alkaline hydrolysates of RNA (11)

Myleran treated animals seem to be unable to recover from the effects of the drug without the aid of exogenous erythropoietin. So it can be assumed that their endogenous supply is either insufficient or that alkylating agents inactivate or inhibit the production of erythropoietin. Using the Ouchterlony immunological technique (15) we were unable to demonstrate the presence of erythropoietin in sera of animals treated with Myleran even when they had been given protective doses of erythropoietin. GALTON's suggestion that Myleran could inactivate a 'substance necessary for cell division by inactivating or blocking its synthesis might actually be the case. It has been reported by FISHER et al. (16) that mercaptomerin treated animals are unable to respond erythropoietically to cobalt. Since the former inactivates sulfhydryl groups (as Myleran might) it can be concluded that it either inactivates erythropoietin itself or inhibits enzymes necessary for erythropoietin production. Further work by FISHER et al. (17) demonstrated the inability of dogs to respond to cobalt after *in situ* kidney perfusion with Myleran. The conclusion gleaned from these data that Myleran interefers with a specific factor is at present difficult to correlate with its nonspecific effects on various cell types.

Chronic toxicity studies of various dosages of Myleran show that the peripheral effects of the drug are directly proportional to dose. This suggests a quantitative inhibition at the molecular level.

Finally it is interesting to compare the percents of marrow lymphocytes in the variously treated groups. Protection of peripheral white cells was afforded by both splenectomy and erythropoietin. This protection was predominantly lymphocytic in the former and neutrophilic in the latter The lowest percent of marrow lymphocytes occurs in erythropoietin treated animals (the high percent in splenectomized animals may be the result of the feedback mechanism mentioned earlier) In the case of erythropoietin, there is a distinct indirect proportion between numbers of circulating red cells and percent of lymphocytes in the marrow

In conclusion, it should be noted that the preceding explanations are based on a plausible, but as yet unproven, theory which rests on the premise that asymmetrical division does not occur in hemopoietic cells. These experiments do not provide quantitative evidence for the stem cell theory but more important, they do not negate it, and are intelligible in light of this theory

Summary

Erythropoietin is significantly protective against the lethal hematological effects of large doses of Myleran. Splenectomy is also protective against the lethal effects of Myleran. The protective effects of erythropoietin are minimized in splenectomized animals. The mechanism of protection of erythropoietin and splenectomy is discussed in reference to bone marrow stem cell kinetics and possible lymphocyte transformation.

Résumé

L érythropoïétine protège de façon certaine contre les effets hématologiques de grandes doses de myleran, de même que la splénectomie. L effet protecteur de l'érythropoïétine est diminué chez les animaux qui ont subi une splénectomie. Le mécanisme de l'effet protecteur de l'érythropoïétine et de la splénectomie est discuté par rapport à la cinétique des cellules-souches de la moëlle osseuse et de leur transformation en lymphocytes.

Zusammenfassung

Erythropoietin schützt vor dem letalen hämatologischen Effekt großer Dosen von Myleran. Denselben Effekt ruft die Splenektomie hervor Die protektive Wirkung von Erythropoietin ist bei splenektomierten Tieren sehr gering. Der Mechanismus der Schutzwirkung von Erythropoietin und Splenektomie wird erörtert im Zusammenhang mit der Kinetik der Stammzellen des Knochenmarkes und der möglichen lymphocytären Transformation.

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Haemoglobin H Disease Among Chinese Residents of Taiwan

By Kuo-Sin Lin, Teh-Chien Lee, Tsung-Cho Lu, Jeanette Tung-Hsiang Huang and R. Quentin Blackwell

The existence of haemoglobin H was reported by Rigas et al. (1, 2) who found it in members of a Chinese family native to Kwangtung Province. At the same time, Gouttas et al. (3) reported its occurrence in Greek subjects. Following those initial studies haemoglobin H was reported in other Greek subjects (4, 5, 6) as well as in several other ethnic groups including Filipinos (7, 8), Malayans (9), Thais (10), Gurkhas (11, 12), Arabs (Trans-Jordan) (13), Iraqui Jews (14), Italians (15) and Sardinians (16). It also was reported to be present as a minor component, along with F and Bart's types of foetal haemoglobins, in cord blood from Negro babies (17).

In addition to the initial Chinese subjects with haemoglobin H studied by Rigas et al. (1, 2, 18, 19), other cases have been reported among Chinese populations in Thailand (20), Indonesia (21, 22), Singapore (23, 24), and Hawaii (25, 26). During the past 3 years studies on Haemoglobin H have been made among Chinese residents of Taiwan; preliminary results of that work are given below.

Methods

Haemoglobin, haematocrit, erythrocyte and reticulocyte counts were determined by standard procedures (27); fetal haemoglobin was estimated according to Kohn et al. (28). The method of Hunter (29) was used to test for erythrocyte osmotic fragility. Inclusion bodies were demonstrated in the RBC (27) by mixing one drop each of 1%

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brilliant cresyl blue and whole blood on microscope slide covering the mixture with cover glass sealed with petroleum jelly and allowing it to stand for t least 30 minutes before microscopic examination. T detect the possibility of sickling preparations were made with equal volumes of whole blood and 2% sodium metabisulfite. Electrophoretic mobilities of the haemoglobins were studied with the Smithies vertical starch gel procedure (30) at pH 6.5, with modified citrate buffer (31) and at pH 9.0 with GOLDBERG TRIS-EDTA-borate buffer (32). The latter buffer system is useful for detecting A_2 haemoglobin as well as showing clearly the rapid mobility of haemoglobin H. Further identification studies were made with paper electrophoresis using the Spinco Model RD-2 power supply with the Durrum type cell. Runs were made with the pH 6.5 citrate buffer (31), with pH 7.0 and 7.5 phosphate buffers, with HCl-TRIS buffer at pH 8.0, with pH 8.5 barbiturate buffer and with the GOLDBERG buffer (32) pH 9.0. An authentic sample of haemoglobin A+H from Thai patient, kindly mad available by Dr PRAWASE WASI, Siriraj Hospital, Bangkok, Thailand, was used for comparison purposes.

Subjects

The present report concerns 6 cases of haemoglobin H disease among 5 Chinese families in Taiwan. Five of the 6 cases, 3 males and 2 females, were children; the remaining one (case 5) was 23-year-old male. Case 1 9-year-old male, was studied less than the others because of his death; Case 6 was younger sister of case 1 Four of the 5 families were native to Fukien and residents of Taiwan and had members available for study: none of the family of case 5, who was native to Kwangtung Province, were in Taiwan.

Case 1 KKH, 9-year-old boy nativ of Fukien, was admitted to the Paediatric Ward of the National Taiwan University Hospital (NTUH) on October 27 1961 because of pallor and dyspnoea. He had been noted to have pallor since 7 months of age and was first hospitalized in May 1957 t the Paediatric Ward of the NTUH. On the second admission the patient' body weight was 19 kg and his height was 115 cm. The only abnormal physical signs were pallor slightly icteric sclerae engorgement of cervical veins and hepatosplenomegaly (liver was 7 cm and spleen 11 cm palpable below the costal margins) Main findings are shown on tables I and II. Bone marrow showed hyperplasia of erythroid precursors. Skull X-ray was normal. During the second admission, total of 900 ml of blood was transfused. He died of pneumonia t home in January 1962, about 2 months after discharge

Case 2: LEJ 5-year-old boy native of Fukien, was admitted to the Paediatric Ward of the NTUH on February 16, 1962, with the chief complaint of attacks of dyspnoea which had been occurring frequently for more than one year His body weight was 16.5 kg and his height was 105 cm. Although he looked pale the conjunctivae showed no signs of anaemia or icterus. Liver and spleen were not palpable. The rest of physical examination was negative. Main laboratory findings are shown in table II

Case 3. WCJ 5-year-old boy native of Fukien, was brought to the Paediatric Out-patient Clinic of the NTUH on March 5, 1962, because of pallor which had persisted since one year of age. Body weight was [illegible] kg and height was 93 cm. There were no abnormal physical signs except slight pallor The main laboratory findings are shown on table II. Bone marrow showed erythroblastic hyperplasia. Iron and pyridoxine preparations had no effect on the anaemia.

Case 4 YCT 13-year-old girl, native of Fukien, was brought to the Paediatric Out-patient Clinic of the NTUH on April [illegible], 1963, with the chief complaints of persistent pallor since early childhood, easy fatigability and stunted growth. Her body weight was 24 kg and her height was 124 cm. The only abnormal physical signs were pallor slightly icteric sclerae and hepatosplenomegaly (both liver and spleen were palpable

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Methods

Haemoglobin, haematocrit, erythrocyte and reticulocyte counts were determined by standard procedures (27) fetal haemoglobin was estimated according to Singer et al. (28). The method of Hunter (29) was used to test for erythrocyte osmotic fragility. Inclusion bodies were demonstrated in the RBC (27) by mixing one drop each of 1%

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Table II

Summary of laboratory data in haemoglobin H disease cases.

Case Number Patient Age in years, Sex	1 KKH 9, M	2 LEJ 8, M	3 WCJ 5, M	4 YOT 13, F	5 CMC 23, M	6 KLC 6, F
b., g%	2.9	11 4	8.7	7 4	13.6	6.2
oetal Hb, %		2.0	2 6	3.4	2.4	2.3
aematocrit, %	15	38	31	29	46	25
BC, 10^6	1.0	5 6	4 7	4.3	6 2	2.9
CV μ^3		68	66	67	74	86
CH, $\mu\mu g$		20	19	17	22	[illegible]
CHC, %		30	28	26	30	25
BC	2950	7600	13000	5600	10000	8600
eticulocytes, %	14.5	2 7	4.8	9.3	6.5	10.1
rum bilirubin, mg%	1.2/2.6		0 05/0.6	0.2/1.6	0.2/1.6	
BC osmotic fragility	decr		decr	decr	decr	
BC inclusion bodies	+	+	+	+	+	+
arget cells	+	+	+	+	+	+
oikilocytes	+	+	+	+	+	+
owers' test	—		—	—	—	

Extremely low values for haemoglobin, haematocrit, and RBC in case 1 preclude reliable mates of MCV MCH, and MCHC.

Determinations not made

23 year old male, had a haemoglobin value in the normal range, 13 6 g% his blood picture was nearly normal except for the presence of the fast moving H component and the poikilocytosis, target cells, and inclusion bodies. The RBC counts in cases 1 and 6 who were brother and sister were markedly reduced while those in the remaining cases were normal. Cases 2—5 exhibited varying degrees of microcytic anaemia case 6 KLC, with an MCV of 86 appeared to have a normocytic anaemia. For case 1 KKH, no reliable estimates of MCV MCH, and MCHC could be made because of the severity of the anaemia. Four patients were tested for osmotic fragility and all showed an increased resistance to hypotonic saline. Inclusion bodies, target cells, and poikilocytes were present in all cases. Five of the patients, cases 2—6 were tested for foetal haemoglobin and in all the amounts were within normal limits.

Based on inspection of the starch gel electrophoretic patterns run at pH 9 0 (32) none of the patients showed evidence of elevated levels of A_2 haemoglobin. No quantitative analyses for haemoglobin H content were made however from observation of electrophoretic patterns of the freshly collected specimens, none appeared to con-

Table I
Summary of principal signs and symptoms in haemoglobin H disease cases.

Case Number Patient Age in years, Sex	1 KKH 9 M	2 LEJ 5, M	3 WCJ 5, M	4 YCT 18, F	5 CMC 23, M	6 KLC 6, F
lenomegaly	+	—	—	+	+	+
epatomegaly	+	—	—	+	+	+
dema	+	—	—	—	—	—
undice	+	—	—	+	+	—
allor	+	+	+	+	+	+
yspnea	+	+	—	—	—	—
sy fatigability	+	+	—	+	+	—
rowth and physical	Normal	Normal	Normal	Below Normal	Below Normal	Normal
stimated age at which onset of allor was noted	7 months	3 years	1 to 2 years	3 to 5 years	6 to 10 years	Unknown

3.5 cm below the costal margins). Main laboratory results are shown on table II. Skull X-ray revealed coarse bone trabeculae.

Case 5. CMC, 23-year-old male medical student, native of Kwangtung, first visited the Medical Clinic of the NTUH on April 4 1963, because of an appearance of pallor which had persisted since he was 2 years old and had not responded to various haematonics. He had experienced several episodes of jaundice in childhood and had been treated as "liver disease. His body weight was 51 kg and his height was 168 cm. The only abnormal physical signs were pallor slightly icteric sclerae and hepatosplenomegaly (liver was 2.5 cm and spleen 0.5 cm below the costal margins). The main laboratory findings are shown on table II. His family lived in Hongkong and were unavailable for haematological studies.

Case 6. KLC, 6-year-old girl, native of Fukien, was younger sister of case 1. She had been doing well since birth in spite of moderate anaemia and hepatosplenomegaly first detected during family studies of case 1. According to her parents there were no episodes of jaundice, dyspnea or other disabilities attributable to the anaemia. The clinical signs and main laboratory findings are summarized in tables I and II.

Results

Typical signs and symptoms in the 6 patients are summarized in table I. Four of the 6 patients exhibited hepato-splenomegaly in 3 of those same patients jaundice was apparent. Pallor was apparent in all patients however the estimated age of onset was variable. Dyspnea was reported in 2 patients. Physical development was considered to be within normal limits in 4 patients and somewhat below normal in the remaining 2 patients.

The results of laboratory findings in the 6 cases are summarized in table II The 5 children whose haemoglobin values ranged from 2.9 to 11 4 g ‰ exhibited varying degrees of anaemia. Case 5, a

the occurrence of the electrophoretically fast H haemoglobin component slight elevation in the haemoglobin F normal amounts of haemoglobin A_2; diminished red cell osmotic fragility and the presence of red cell inclusion bodies, target cells, poikilocytosis, and reticulocytosis. The patients exhibited varying degrees of anaemia which in some cases caused little disability but which in one patient was sufficiently severe to cause death. The anaemias were microcytic and hypochromic in 4 of the 6 cases. The severity of the anaemia in the patient who died made classification difficult. The sixth case, sister of the patient who died, had normocytic hypochromic anaemia.

Résumé

Six cas de maladie à hémoglobine H, d'une famille chinoise de Taïwan, sont étudiés. Les résultats des examens hématologiques ressemblent à ceux de rapports précédents, y compris la présence d'une composante d'hémoglobine H rapide à l'électrophorèse, une légère augmentation de l'hémoglobine F une quantité normale d'hémoglobine A_2, une diminution de la fragilité osmotique des érythrocytes ainsi que la présence d'inclusions érythrocytaires, de cellules en cibles, de poïkilocytose et de réticulocytose. Les malades montraient différents degrés d'anémie qui dans certains cas ne causait que peu de troubles mais qui, chez un malade, était si grave que la mort en suivit. Les anémies étaient microcytaires et hypochromes dans 4 des 5 cas. La gravité de l'anémie chez le malade décédé est difficile à classer. Le sixième cas, une soeur du malade décédé, avait une anémie normocytaire hypochrome

Zusammenfassung

Es werden 6 Fälle von Hämoglobin-H Krankheit bei 3 chinesischen Familien in Taiwan untersucht. Die hämatologischen Befunde glichen denjenigen früherer Beobachtungen einschließlich des Vorkommens der elektrophoretisch langsam wandernden Hämoglobin-H-Komponente, einer geringen Vermehrung von Hämoglobin F einem normalen Gehalt an Hämoglobin A_2, einer erhöhten osmotischen Erythrocytenresistenz und des Vorkommens von Erythrocyten-Einschlußkörperchen, Target-Zellen, Poikilocytose und Reticulocytose. Die Patienten zeigten eine Anämie in wechselndem Ausmaß, die in einigen Fällen wenig Beschwerden verursachte, bei einem Fall aber so schwer war, daß sie zum Tode führte. Die Anämien waren mikrocytär und hypochrom bei 4 der 6 Fälle. Die Schwere der Anämie bei dem verstorbenen Patienten war schwer zu klassieren. Der 6. Fall, eine Schwester des verstorbenen Patienten, hatte eine normocytäre hypochrome Anämie.

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Thalassemia with Complete Absence of Hemoglobin A_2 in an Adult

By R. B. Thompson, J. Odom, R. Warrington and W. N. Bell

Over the past few years considerable emphasis for the sometimes difficult diagnosis of thalassemia minor has been placed on the elevation of hemoglobin A_2, the minor component of normal adult hemoglobin. A two fold increase of the normal percentage of hemoglobin A_2 occurs in the usual type of beta chain thalassemia trait or in hemoglobin H disease (1). A decrease of the hemoglobin A_2 fraction down to 1% has been observed in some alpha thalassemias (HbH) (7) or as a reversible phenomena in iron deficiency anemia (9). Traces of hemoglobin A_2 can be detected in cord blood hemoglobin by the application of special techniques (5). However only one case report has appeared in the literature in which the features of thalassemia were present but in which there was complete absence of hemoglobin A_2 (4). Because of similar findings in an 82 year old white male the following paper was written.

Methods

Peripheral blood smears stained with Wright's stain were examined. The osmotic resistance of the red cells was determined by techniques as outlined earlier (8). The preparation of hemoglobin solutions for electrophoresis was by the method of Thompson et al. (8). The clear hemolysate was subjected to electrophoresis on starch gel prepared as outlined earlier (5). Alkali denaturation was performed by the two minute procedure of Chernoff et al. (3). Quantitation of hemoglobin fractions was done on DEAE cellulose chromatography as outlined by Huisman et al. (6). For measurement of ferrokinetics, the method of Brodine and Dettman (2) was used.

Case Report

An 82 year old white male (W. W. No. 94864) was admitted to the University of Mississippi Medical Center on the 26th of November 1963 with history of anemia

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requiring frequent transfusions over several years. Physical examination revealed well-developed, pale, white male, blood pressure 130/70 pulse 86 and temperature 99 °F There was no icterus. Neither liver spleen were palpable.

Hematologic investigation: Hemoglobin 7.4 g%, hematocrit 25 reticulocytes 2.2% and RBC 3,600,000. Wright stained peripheral smears showed hypochromia 3+ polychromasia 1+ occasional poikilocytes, microcytosis 1+ and anisocytosis 2+ Target cells were numerous (fig. 1) Serum iron was 150 µg% and iron binding capacity 450 µg%.



Fig 1 Peripheral Wright's stained blood smear Numerous target cells are present. 950 ×

Hemoglobin electrophoresis (fig. 2) showed the presence of hemoglobin A only; hemoglobin F amounted to less than 2.0%. Quantitation of the hemoglobin fraction on DEAE cellulose chromatography again showed only hemoglobin A.

Bone marrow biopsy Hyperplastic marrow with an M E ratio of 1.9 1 The red cells showed considerable microcytosis, hypochromia, and pproximately 30% target cells. Nucleated cell distribution was as follows Promyelocytes 1% myelocytes 10% metamyelocytes 14% bands 19% segmented neutrophils 13% eosinophils 1%, lymphocytes 13% erythroblasts 3% pronormoblasts 23% normoblasts 4% There was slight reticulocytosis of pproximately 1.5% Osmotic fragility of the erythrocytes was decreased.

Determination of erythrocyte life span Half-life of 26 days Plasma iron clearance was increased to 30 minutes, (normal 60—120 minutes) and iron re-utilization by red cells 14%.

Fasting gastric analysis No free acid no free acid after alcohol and histamine stimulation. 9% renal excretion of Co^{60} in 24 hours was obtained Stools were repeatedly negative for blood.

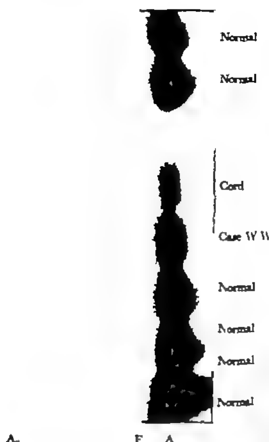


Fig. 2. Electrophoresis on starch gel at pH 8.1 (Benzidine stain). A normal adult showing HbA and A_2 and cord sample with only HbA and F has been run along with case W. W. under study. Absence of HbA_2 is illustrated.

The patient was placed on high protein diet alternating with intramuscular injections of pyridoxine, folic acid and Inferon. No reticulocyte response was elicited after two months of therapy.

No children or siblings were alive for additional study.

Comments

The diagnosis of thalassemia was based on the findings of a low MCV (63 μ^3), a markedly decreased osmotic fragility, the presence of 30 % target cells, a serum iron of 140 μg % and iron binding capacity of 415 μg % plus an anemia refractory to all forms of therapy.

The unexpected findings of a normal Cr^{51} erythrocytic survival in a severely anemic patient with no evidence of blood loss suggests that one is dealing with factors other than those limiting erythrocytic survival. The decreased and incomplete utilization of Fe^{59} by the reticulocytes suggest that perhaps the basic defect in this type of thalassemia lay at the level of incorporation of iron into the protoporphyrin molecule.

As far as we aware this is only the second case of an adult having almost exclusively HbA_1 and entirely lacking HbA_2. Present day evidence suggests that HbA_2 and thence the delta chain is under a separate genetic control with perhaps a close linkage existing between the beta and delta chains. Absence of HbA_2 would be expected in alpha or delta chains abnormalities, but in such cases an abnormal component such as HbPylos, HbBarts or HbF (homozygous persistent high F gene) should replace HbA_2. In the case under discussion as well as in the case reported by FESSAS (4) there was no evidence of an additional or abnormal component. We therefore agree with FESSAS that the genetic lesion causing the complete suppression of HbA_2 must be associated with a gene affecting solely the delta chain production. The individual in this case must be considered as possessing a defect on both delta chains and thus be homozygous for a delta chain defect.

Summary

A patient presenting with severe hypochromic microcytic anemia and complete absence of HbA_2 is presented. The genetic basis for such an unusual type of thalassemia is discussed and the possibility of incomplete iron incorporation in the protoporphyrin molecule is suggested as the cause of the hematologic picture.

Résumé

Description du cas d'un malade présentant une anémie hypochrome microcytaire sévère et une absence complète d'HbA_2. La base génétique de ce type peu commun de thalassémie est discutée. Une incorporation incomplète du fer dans la molécule de protoporphyrine est proposée comme cause du tableau hématologique.

Zusammenfassung

Es wird über einen Patienten mit schwerer hypochromer Anämie und vollständigem Fehlen von HbA_2 berichtet. Die genetische Grundlage für diesen ungewöhnlichen Typ von Thalassämie wird diskutiert. Als Ursache des hämatologischen Bildes wird ein inkompletter Eiseneinbau in das Protoporphyrinmolekül vermutet.

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Varia

Standardization in Haematology

The Panamerican Meeting for Standardization in Haematology will take place from June 14 to 17, 1965, in Ica, Perú (National University "San Luis Gonzaga"). The Peruvian Society of Haematology invites the haematologists of all American countries to support the International Committee for Standardization in Haematology initiating National Standardization Committees according to the plan accepted at the 10th Congress of the International Society of Haematology, Stockholm 1964.

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Deucher/Müller: Der operierte Magen. Bibliotheca Gastroenterologica, Fasc. 6. S. Karger AG, Basel/New York 1964. XX + 278 S., 92 Abb., 63 Tab. Preis sFr./DM 63.–

Das Verhandlungsthema der Jahresversammlung der Schweizerischen Gesellschaft für Gastroenterologie vom 24. bis 26. Oktober 1963 in Solothurn war der operierte Magen. Der vorliegende Band stellt eine Sammlung der von Internisten, Chirurgen und Röntgenologen gehaltenen Vorträge dar. Auf eine anschauliche Darstellung der physiologischen Grundlagen der Resektionstherapie folgen Referate über die Operationsmethoden und ihre Spätresultate, die Untersuchung des operierten Magens, die Syndrome nach Magenresektion und die Behandlung des operierten Magens. Auf eine Erörterung der besonderen Probleme der chirurgischen Therapie der malignen Magenerkrankungen wurde bewusst verzichtet. Insgesamt vermittelt der Band, der zahlreiche hervorragende Beiträge namhafter Fachleute enthält, einen ausgezeichneten Überblick über den heutigen Stand der chirurgischen Behandlung der Ulkuskrankheit. Unterschiedliche Meinungen über den Wert alter und neuer Operationsverfahren werden laut, neue Wege der Nachbehandlung Magenoperierter werden aufgezeichnet und diskutiert. Daß manche der eingeführten neuen Methoden noch der kritischen Wertung bedürfen, wird dem aufmerksamen Leser nicht verborgen bleiben. Allen, die sich über die heutige Situation und Problematik der operativen Behandlung der Ulkuskrankheit orientieren wollen, kann das Studium dieses mit zahlreichen Tabellen und Abbildungen vorzüglich ausgestatteten Buches nur empfohlen werden. R. Ottenjann, Stuttgart

L. M. Tocantins/L. A. Kazal: Blood Coagulation, Hemorrhage and Thrombosis. Methods of Study. Grune & Stratton, New York and London 1964. 532 p. Price $ 17.50.

This book was originally published 1955 under the title "Coagulation of Blood and Methods of Study".

The revised and enlarged second edition was conceived and is dedicated to *L. M. Tocantins*, who recently died. The book brings a collection of methods in the field of blood coagulation, in detail described by more than 90 contributors. It will be of great value for every investigator in this field who wants to have reliable and critical information about the methods. G. Rosenow, New York

William Dameshek/Frederick Gunz: Leukemia. 2. edition. Grune & Stratton, New York/ London 1964. 594 p., Price $ 25.00.

The second edition of this book is revised and enlarged; it has incorporated the progress made since the first edition was published 5 years ago (A. H. 22, 365, 1959). Many changes are noticeable; mentioned here is only the chapter "Pathology of the Leukemic Cell" which gives an excellent survey about the chromosomes and their significance in leukemic conditions.

About 1000 new references are added.

G. Rosenow, New York

Carl V. Moore/Elmer B. Brown: Progress in Hematology. Vol. IV. Grune & Stratton, Inc., New York, N.Y. 309 p., Price $ 13.75.

The new editors of these series have replaced L. M. Tocantins who died recently. This volume contains chapters on Heme Synthesis in Erythroid Cells (S. Granick and R. D. Levere), Methemoglobinemias in Man (E. R. Jaffé and P. Heller), short presentation on Erythropoietin (R. D. Lange and V. Pavlovic-Kentera), Chemistry of the ABH Blood Group Substances (G. Schiffman and D. M. Marcus), Hematologic Aspects of Lead Poisoning (R. C. Griggs), an important presentation on Chloramphenicol Toxicity (A. A. Yunis and G. M. Bloomberg), and an excellent chapter on Myeloma Proteins and Macroglobulinemia (G. M. Bernier and F. W. Putnam); others surveys concern Recent Advances in Acute Leukemia (E. J. Freireich and Emil Frei III), von Willebrand's Disease (E. M. Barrow and John B. Graham) and Platelet and Leukocyte Isoantigens and their Antibodies (N. R. Shulman et al.).

G. Rosenow, New York

Acta haemat. 33. 193–199 (1965)

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Increased Hepatic Uptake of Radioiron in Acute Leukemia

By E. J Walter Bowie, W Newlon Tauxe, J M. Stickney and Joseph M. Kiely

Radioiron (Fe^{59}) and radiochromium (Cr^{51}) studies were made in two fatal cases of acute leukemia. Estimations of the radioactivity in various organs, both in vivo by external counting and at necropsy showed increased uptake of Fe^{59} by the liver This finding has been reported previously (2) but the present cases are of unusual interest because the data suggest that the liver shows an increased avidity for iron.

Methods and Calculations

The procedures and calculations were those described by Owen (5) except that heparin was used as the anticoagulant for tagging with Fe^{59} Twelve ml of the patient's heparinized blood was injected into a rubber-capped vial containing the previously measured dose of ferrous citrate, F $(C_6H_5O\ )_2$. After 15 minutes of incubation, 10 ml of the Fe^{59}-tagged blood was injected into the patient by two-syringe technic. Four heparinized samples of blood were withdrawn during the next hour for the determination of the plasma iron clearance rate. The serum iron was measured by the method of Ramsay (7 8) and the iron-binding capacity by previously described technic (9)

Calculation of Radioiron Results. Plasma Fe^{59} Half Clearance Time. Four samples of blood drawn during the 80 minutes after the injection of iron were centrifuged, and the radioactivity of 1 to 3 ml aliquot of plasma was determined in well-type scintillation counter The radioactivity (counts per second per ml of plasma) after correction for background was plotted on semilogarithmic paper with the arithmetic axis used for time. A straight line was drawn through the points and extended to the vertical axis, the intercept of which allowed the calculation of the plasma volume. The time at which half the radioactivity of the iron had disappeared from the plasma was noted. This half time ($t\frac{1}{2}$) was converted into disappearance rate by the formula $\frac{0.693}{t\frac{1}{2}}$

The turnover of plasma iron per day was calculated by multiplying disappearance rate of plasma iron per hour by the total amount of stable iron in the plasmatic pool. This value was then multiplied by 24 to give the plasma iron turnover rate in milligrams per day It was also expressed as mg per 100 ml of whole blood, after the method of Bothwell et al. (1).

Estimation of radioactivity of tissue obtained at necropsy was made on duplicate weighed specimens. Net Fe^{59} and Cr^{51} radioactivity per gram of tissue was determined, and the percentage of the injected dose in the whole organ was calculated.

Report of Cases

Case 1 A 48-year-old man came to the Mayo Clinic in November 1959 with a 2-month history of weakness, anorexia, fever and anemia. The tip of the spleen was palpable, and ecchymoses were present in both antecubital fossae. A diagnosis of acute lymphoblastic leukemia was made by bone marrow examination.

Treatment with chlorambucil had been started elsewhere in September and continued for 8 days, when this agent was replaced by methylprednisolone. On November 19 treatment was started with 150 mg of 6-mercaptopurine daily and 10 mg of prednisone three times daily. These drugs were given in varying dosage until the patient's death, at which time a total dose of 6450 mg of 6-mercaptopurine and 2860 mg of prednisone had been given. In October the leukocyte count had been 4900 cells per mm of blood, with 28% blast cells. By the middle of December the marrow showed improvement, and evidence of immaturity had disappeared from the peripheral blood. The patient remained anemic, however, and required 27 pints of blood in 4 months.

A diagnosis of diabetes mellitus was made on January 13, 1960, and the patient was treated with a 1500-calorie diet and tolbutamide.

A few days before the patient' death the leukocyte count was 1300 with 42% blast cells. He died on February 3, 14 days after the beginning of the Fe^{59} and Cr^{51} studies.

At necropsy the liver kidneys, testes, and bone marrow were extensively infiltrated with leukemic cells.

Case 2. A 29-year-old man came to the Mayo Clinic in April, 1959. He had noted fatigue and exertional dyspnea for a year and buccal ulceration for 9 months. A linear hemorrhage was present below the right optic disk. The liver and spleen were not palpable. Examination of the bone marrow revealed acute myeloblastic leukemia.

The leukocyte count was 17,700 per mm of blood, with 60% blast cells, and the hemoglobin was 7.0 gm per 100 ml of blood. The patient remained anemic throughout the course of his illness, requiring 10 units of blood. Treatment was started with amethopterin (methotrexate) 5 mg daily and 6-mercaptopurine, 250 mg daily. The peripheral blood picture improved, and the leukocyte count declined.

On June, 17 the leukocyte count was only 600, with 1% blast cells. Treatment was then changed to cyclophosphamide and prednisone. Subjective improvement occurred, but the percentage of blast cells in the peripheral blood increased. At the end of July course of therapy with methylglyoxal bisguanylhydrazone sulfate (methyl GAG) was started. This medication caused diarrhea, and the patient was unable to take it in adequate doses.

He was admitted to the hospital on December 1 because of a perianal hematoma and fever. Use of methyl GAG was stopped, and he was given imidazolyl thioguanine (ITG)

The patient' temperature increased to 106° F and he died on December 16. Necropsy disclosed extensive leukoblastic infiltration of the kidneys, lungs and liver. Bilateral bronchopneumonia was present.

Results

The results of the Fe^{59} studies are shown in table I. In both patients there was a great increase in the plasma iron turnover rate, although the erythrocyte incorporation of Fe^{59} was reduced

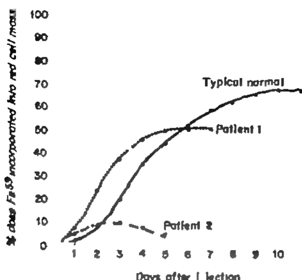


Fig 1 Incorporation of Fe^{59} into red cells was reduced in both patients.

Table I
Fe^{59} and Cr^{51} data in two patients with acute leukemia.

Age, Sex	Normal	Patient 1 48, M	Patient 2 23, M
Serum iron, µg/ml	0.75–1 75	2.47	1.52
Total iron-binding capacity µg/ml of serum	2.75–4 0	2.80	1 79
Latent iron-binding capacity µg/ml of serum	1.5–2.5	0.33	0.27
Amount of inorganic iron added, µg/ml of plasma		0.21	0.18
Plasma Fe^{59} clearance T½, min.	60–120	206	163
Plasma iron turnover mg/day (total)	20–33	34	59
mg/100 ml blood/day	0.42–0.72	0.85	0.70
Erythrocyte-labeled Fe^{59} % of dose	65–80	50	10
Cr^{51} % fall/day	<2.0	3.4	4.9

(fig 1) These findings were especially pronounced in patient 2 whose plasma iron turnover rate was twice normal whereas his incorporation of Fe^{59} into the red cells was only 10 % of the injected dose (normal 70 to 80 %)

In both patients the Cr^{51} disappearance was higher than normal. The results of external scintillation counting (corrected for

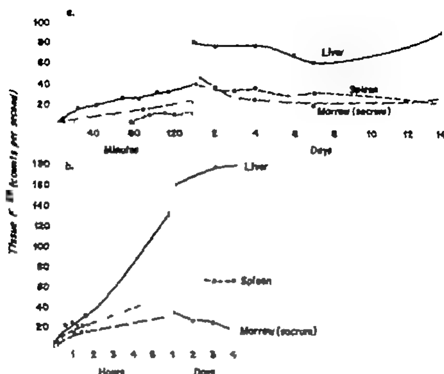


Fig. 2. a) case 1 and b) case 2. Parenchymal Fe^{59} uptake over organs as measured by external body counting. In contrast to the normal situation, iron uptake is much greater over the liver in both cases than over either spleen or marrow. (From Bowe, E. J. W.; Kiely, J. M.; Tauxe, W. N. and Stickney, J. M.: The Anemia of Leukemia. J. nuclear Med. 3: 423–435 (Nov.) 1962. By permission of Samuel N. Turiel and Ass., Inc.).

radioactivity in the blood flowing through the area) are shown in figure 2. There is a striking increase of Fe^{59} radioactivity in the hepatic region.

Organ counts at postmortem examination supported the in vivo findings of increased hepatic radioactivity and are shown in table II. Histologic sections of the liver showed large deposits of iron in both parenchymal and reticuloendothelial cells.

Bone marrow differential counts are given in table III. The percentage of normoblasts was reduced.

Comment

The increased hepatic uptake of iron in certain cases of acute leukemia has never been satisfactorily explained. When erythro-

Table II

Organ radioactivity (postmortem)

Patient	Liver % of dose		Spleen % of dose	
	Fe^{59}	Cr^{51}	Fe^{59}	Cr^{51}
1	54	8	4	8
	(wt. 2820 gm)		(wt. 290 gm)	
2	70	25	10	8
	(wt. 2970 gm)		(wt. 905 gm)	

Table III

Cell differential counts (per cent from bone marrow).

	Normal	Patient	Patient 2
Neutrophils	44.5	11.0	2.5
Lymphocytes	9.5	10.5	18.5
Monocytes	1.5	2 0	4.5
Eosinophils	3.5	0.5	
Basophils	1.0	0.5	
Metamyelocytes	10.0	4.0	
Late myelocytes	6.5	4.0	1.0
Early myelocytes		3.0	2.5
Progranulocytes	2.5	3.0	6.0
Blasts	1.0	46.0	35.5
Immature lymphocytes		7.0	9.5
Atypical lymphocytes		2.5	4.0
Immature monocytes			4.5
Normoblasts	19.0	3.5	7.0
Plasma cells	1.0		3.5
Mitotic megakaryocytes		0.5	0.5
Histiocytes			0.5

poiesis is depressed, the iron may be distributed to the storage sites, the most important of which is the liver and NATHAN AND BERLIN (4) have suggested that some of the hepatic iron is first taken up by the bone marrow (even when erythropoiesis is absent) and is secondarily deposited in the liver On the other hand, an increased hepatic avidity for iron would also explain the findings and is supported by the data obtained from the present patients.

A primary deposition of iron in the liver is suggested by the early and rapid rise of the Fe^{59} activity recorded by external scan-

tillation counting Histologic examination showed no evidence of extramedullary erythropoiesis. It is known that when the iron-binding capacity has been exceeded by the addition of iron containing the label, there is also a rapid primary hepatic uptake of iron. This results in a plasma clearance which is usually more rapid than normal and in unreasonably high plasma volumes, neither of which was observed in these cases. Moreover in neither case did the total iron added exceed the latent iron-binding capacity (table I)

The clearance of iron from the plasma is usually a two-component exponential system with a slower phase following the initial more rapid clearance. POLLYCOVE (6) has demonstrated three components in hemochromatosis. The concept usually presented is that of a system of interconnecting pools with free interchange of iron between them. The two most important factors affecting the turnover rate are the rate of erythropoiesis and the avidity of the plasma iron stores (1)

In the present study the plasma iron turnover rate was increased, but the Fe^{59} incorporation was reduced, a situation often called 'ineffective erythropoiesis In spite of the increased iron turnover rate in these two patients, there was no morphologic evidence of increased erythropoiesis. In both patients the percentage of normoblasts in the marrow was reduced. These findings differ from those in other cases of depressed marrow function, such as hypoplastic anemia, where the plasma iron turnover rates are less than normal.

The morphologic findings together with the decreased erythrocyte incorporation of Fe^{59} in these two cases suggest therefore that the increased iron turnover rate may be explained by a peculiar increase in the avidity of the liver for iron.

GILLMAN et al. (3) have suggested that an abnormality of intracellular metabolism may explain the dietary siderosis in malnourished rats. Such an abnormality has never been suggested in acute leukemia, but it is a possibility that warrants further consideration.

Summary

Studies in two men with acute leukemia showed an increased hepatic uptake of iron. Evidence is presented to show that the liver has an increased avidity for iron in this disease.

Résumé

Des études faites chez deux malades ayant une leucémie aiguë démontrèrent une absorption augmentée de fer par le foie. Une avidité accrue du foie pour le fer est mise en évidence dans cette maladie.

Zusammenfassung

Bei zwei Patienten mit akuter Leukämie fand sich eine erhöhte Eisenaufnahme in der Leber. Es wird nachgewiesen, daß die Leber bei dieser Krankheit eine gesteigerte Avidität für Eisen besitzt.

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Increased Absorption of Radioiron in Gastrectomized Patients by the Addition of Hydrochloric Acid

By S. Moeschlin, J. R. Schmid and Th. Schneider, Solothurn

Hypochromic microcytic anaemia is a fairly frequent finding in gastrectomized patients while macrocytic forms are rare (1 to 7). The incidence of iron deficiency anaemias in these patients differs considerably in larger series (4, 7). While Wells et al. (8) noted anaemias in only 11 %, Blake et al. (5) found it in as many as 40 % of their cases. In all these studies, hypochromic anaemias were more commonly encountered in females than in males, the ratio being e. g. 20 % : 9,5 % in one study (4). The incidence remains higher for women beyond the menopause. In general, iron deficiency anaemias in non gastrectomized males are rare and, if present, usually due to blood loss, malignancy or infection.

Sideropenic anaemias in gastrectomized subjects present morphologically identical findings as hypochromic anaemias of other cause. In the bone marrow there is also an increase of immature normoblastic forms, especially of the large macroblasts corresponding to Wicker's (9) K_1 stage. *Ferritin iron may be absent in* the macrophages of the marrow. Reticulocytes are reduced in number if therapy has not yet been instituted and if there is no blood loss.

Factors inducing sideropenic anaemias in gastrectomized subjects. By the use of precise methods of study, a decrease in iron absorption is commonly found (2, 3, 10 to 12). In addition to such a decreased rate of absorption, Stevens (10) presented evidence that patients with depleted iron stores are unable to compensate this lack by an increase in absorption following gastrectomy. Normal losses of iron amount to 0.5 to 1.5 mg per day while 1 to 2 mg of iron is absorbed in the gastrointestinal tract, primarily from animal sources (13). During the menstrual cycle iron loss is considerably enhanced in females which are more prone to develop a deficiency for this

reason. There are a number of pathogenetic factors that may at times in combination, induce a deficiency state, primarily of iron at times also of folic acid and, exceptionally also of vitamin B_{12}.

Factors leading to a decrease in absorption following partial (Billroth II) gastrectomy 1 HCl-deficiency (deficiency of pepsin?) 2. Decreased absorptive surface (in stomach and duodenum) 3 Decreased mixing of food with bile and pancreatic enzymes 4 Bacterial invasion of the duodenum 5 Nutritional imbalancies (diet) 6. Diminished intake in vitamin C (intolerance to fruits) — In the experiments to be described subsequently the role of the first mentioned factor shall be investigated in detail in partially gastrectomized patients. To exclude conditions of abnormal blood loss, only males with no evidence of bleeding were selected for the study The haemoglobin level ranged from 10.5 to 15 0 g %. All patients studied presented gastric achlorhydria.

It was the purpose of this study to evaluate whether the decreased iron absorption may be improved by the addition of hydrochloric acid or pepsin in gastrectomized individuals. Fe^{59}-citrate, 5 μc with a total dose of 0.7 mg of iron, was administered to the fasting patient. Subsequently the postabsorptive increase of the radioactivity was followed over a 6-hour period in the plasma. By this procedure, the per cent absorption of the oral dose may not be calculated. However it is possible by this method to study the influence of different factors on iron absorption by comparing the different postabsorptive curves in the same patient.

Method

Following 12-hour fasting interval, blood was withdrawn for the determination of serum iron and latent iron binding capacity (method of TAUXE, 14) The patient was then given 5 μc of Fe^{59}-citrate with dose of 0.7 mg orally Neither ascorbic acid, food or large doses of iron salts were added. The radioactivity was then measured in the plasma at regular intervals up to 6 hours in 2 inch sodium iodide (Tl) Well type scintillation counter After determination of the Cr^{51} plasma volume, the obtained values were expressed as cpm. for the total plasma volume. A second study was performed at time the radioactivity had dropped to zero in the plasma. The patient then received an identical dose of Fe^{59}-citrate with simultaneous addition of 30 drops of 0.1 HCl In third experiment, 0.5 g of Pepsinum Ph. H. was added and in fourth study 0.5 g of Pepsinum Ph. H. was administered in combination with 30 drops of 0.1 HCl.

Results

Fe^{59} absorption curves in 6 healthy males. The postabsorptive curves are characterized by a fairly rapid initial increase with a peak of

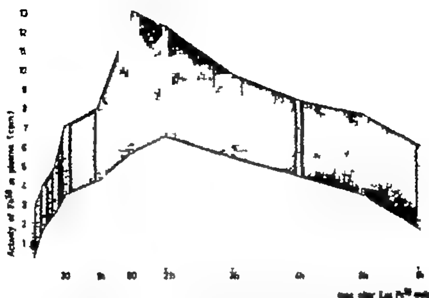


Fig. 1 Postabsorption curves of Fe^{59} in 6 healthy males after 5µc of Fe^{59}-citrate orally and a dose of 700 µg of iron. No addition of ascorbic acid. The peaks of absorption are reached after 90 minutes with a gradual subsequent drop-off. (Ordinate: plasma radioactivity abscissa: time after the oral dose of radioiron.)

Table I
Data of the 6 healthy male subjects.

Name		Hb g%	Serum iron µg%	Latent iron binding capacity µg%	Fe^{59} peak of absorption curve (cpm/plasma ml.)
1 M. V.	1937	15.0	76	176	130 [illegible]
2 M. M.,	1931	15.9	90	221	106 700
3 J. F.	1918	13.2	108		82 900
4 L. H.,	1942	13.2	134	169	94 700
5. R. O.,	1921	15.4	136	133	78 400
6. S. J.	1941	14.8	152	204	63 900

the radioactivity reached after 90 to 120 minutes. There is a subsequent gradual decrease due to the outflow of iron to the tissues, primarily the bone marrow and a gradual diminution of iron absorption (fig. 1). In fig. 2 the peak values of the postabsorptive curves are plotted with the measured values for serum iron in 6 healthy male patients (table I). It may be noted that the highest absorption peaks were found in patients with relatively low serum iron levels and vice versa.

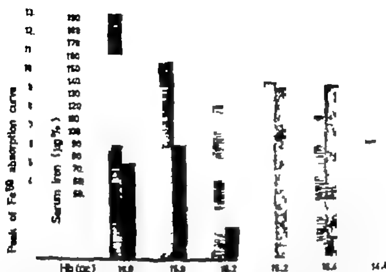


Fig. 2. Relationship between the peak values of the Fe^{59} absorption curves and serum iron in 6 normal male subjects. The highest absorption peaks are found even in healthy individuals in the presence of rather low serum iron level and vice versa. It is conceivable that even normally there is reciprocal influence between the serum iron level and iron absorption from the gut.

Fe^{59} absorption curves in 6 male patients after subtotal (Billroth II) gastrectomy before and after the combined addition of hydrochloric acid and pepsin. The postabsorptive curve prior to the addition of HCl and pepsin is low and flat compared with the one obtained in normal controls. The peak of absorption is lower although it is already obtained after an interval of 30 minutes. There is marked delay in the subsequent drop-off of the radioactivity. The combined addition of HCl and pepsin distinctly increases absorption of radioiron without reaching values as those found in the controls (fig. 3).

Fe^{59} absorption in 5 male patients with and without the addition of pepsin alone. The influence of pepsin on iron absorption may be recognized from fig. 4. Pepsin markedly inhibits iron absorption. This action of pepsin may be compared with the well known inhibitory effect of pancreatic enzymes on iron absorption (15, 15a).

Fe^{59} absorption with and without the addition of hydrochloric acid alone. Experiments presented in fig. 5 reveal that 9 male patients with subtotal gastrectomy exhibit in general an increase of Fe^{59} absorption by the administration of HCl. The average values of the plasma radioactivity is increased by more than twice by HCl and

3.

4 3 2 1

Activity of Fe^{59} in plasma

30' 1h 90' 2h 3h 4h 5h

hours after ingestion

Fig. 3. Individual and average values of the Fe^{59} postabsorptive activity in the plasma in 6 partially gastrectomized patients. The difference in the mean values indicates the marked increase of iron absorption obtained by simultaneous addition of HCl and pepsin.

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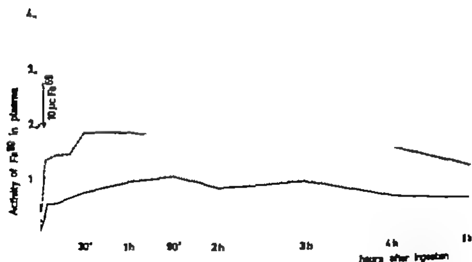


Fig. 4 Distinct inhibitory effect on the oral Fe^{59}-citrate postabsorptive curves by the addition of pepsin.

- - - - prior to the addition of pepsin ——— following the addition of pepsin.

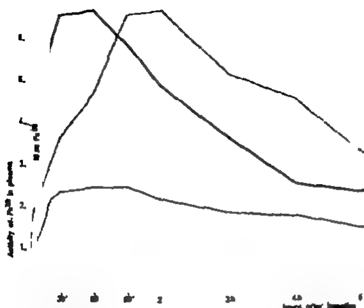


Fig. 5. Normal absorption curves of Fe^{59}-citrate in 6 healthy individuals in comparison with the very low values obtained in 9 patients gastrectomized by the Billroth II procedure. By the addition of HCl, the Fe^{59} absorption curve becomes practically normalized, with the exception that the peak values are reached after 30 minutes compared with 90 to 120 minutes in the controls.

6 normal subjects without HCl ——— Billroth II without HCl (9) ———
Billroth II with HCl (9) ———

approach those of 6 control subjects without addition of HCl. The main difference between these two groups relates to the fact that the peak values are already obtained about 30 minutes after the oral dose, a finding to be interpreted most likely by the enhanced passage of food into the jejunum following gastrectomy. Conversely peak values in normals are obtained after an interval of 90 to 120 minutes, only. An illustrating example of the effect of HCl in a patient with previous sideropenic anemia is given in fig. 6.

Discussion

In a previous extensive study (16) using the combined administration of hydrochloric acid and pepsin only, the different factors influencing the postabsorptive Fe^{59} curve have been discussed. The principal ones are: plasma volume, amount of depot iron, erythropoietic activity of the marrow, plasma clearance of iron, total dose

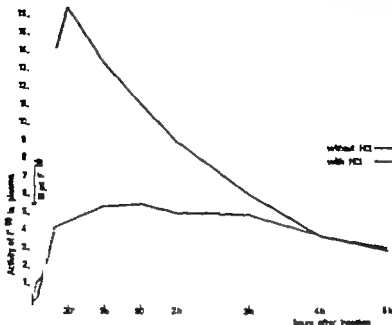


Fig. 6. Illustrating example of recurrent, sideropenic, hypochromic anaemia in patient operated 1960 by the Billroth II procedure. Prior to the study the patient was treated with iron and the haemoglobin level rose from 7.3 to 13.4 g % and the serum iron level became stabilized at 74 µg %. At this time study of iron absorption revealed three-fold increase obtained by the addition of HCl to the oral Fe^{59}-citrate dose.

of Fe^{59} and relation to non-radioactive iron administered, mode of application, valence of iron, inorganic or food iron, levels of serum iron, latent iron binding capacity and sex of the patient. The factors plasma volume and sex of the patient could be eliminated: the plasma volume was measured in each instance (by the Cr^{51}-method, 17, 18) and only male patients were selected for the study. In the control group routine blood studies and also the clearance rate of radioiron (19, 20) were within normal limits in each instance. Fairly steady state conditions may, therefore, be assumed for these subjects.

The oral dose administered is certainly of great significance in such a study (21 to 23). Smith et al. (23) were able to show that the use of a very small dose of carrier iron, similar to the one used in the present study, gives an absorptive rate in normals of about 30 %. The addition of ascorbic acid may increase absorption to as high as 70 % (22). By the addition of larger doses of non-radioactive

iron, the amount absorbed may drop as low as 10 %. These same authors (22) demonstrated further that postmenopausal females with normal haemoglobin and serum iron levels absorbed iron at a higher rate than males.

The present studies (fig 2 and table I) present new evidence to assume that Fe^{59} absorption may even in normal persons be related to variations of the daily and individual level of serum iron. There seems to be a reciprocal relationship between level of serum iron and degree of iron absorption. However no relationship was recognized in our six healthy controls between absorption of Fe^{59}-citrate (peak values) and latent iron binding capacity of the plasma (table I) which has been postulated by HALLBERG et al. (20, 24) Transferrin levels (19 25) were not determined directly however in our cases.

Our studies with Fe^{59}-citrate gave average absorption peak values in controls in the order of 6.5—13.5×10^4 cpm while those of gastrectomized patients were 1.5—4.5×10^4 cpm (fig 3) After gastrectomy iron absorption was found to be more rapid initially probably due to enhanced passage of food into the gut.

With the addition of HCl a marked increase in Fe^{59} absorption, in the order of 2 or 3-fold, was observed in partially gastrectomized patients (fig 5 and 6) It appears likely from these studies that Fe^{59} absorption may be normalized in patients with Billroth II procedures by the addition of HCl. Pepsin, on the other hand, seems to inhibit Fe^{59} absorption (fig 4) This finding is well in agreement with the first one noted, in which the combined administration of HCl and pepsin gave lower peak values of iron absorption than the application of HCl alone. Our investigations thus confirm findings of GOLDBERG (26) in patients with gastric achylia. In the treatment of patients with sideropenic, hypochromic anemia after gastrectomy the administration of hydrochloric acid may be advisable, in combination with oral iron preparations.

The following directives may be recommended for prophylaxis and therapy of the anaemia in gastrectomized patients

Prophylaxis Total gastrectomy resection of the gastric fundus (B_{12}) and stasis in the afferent loop should be avoided.

Therapy Diet rich in iron (meat!) HCl 0 1 n., 30 to 50 gtt. to each meal oral and, if necessary intravenous iron preparations. Folic acid only in the presence of macrocytic anaemia or early marrow changes (coarse chromatin structure of early normoblastic

forms) 15 mg of folic acid i. m. daily Vitamin B_{12} only in the presence of pernicious anaemia, which is extremely rare (16)

Summary

The importance of deficiency in hydrochloric acid was investigated in partially gastrectomized male patients without anaemia by following the postabsorptive plasma activity of radioiron (Fe^{59}-citrate). Comparison of the results with those found in normal males showed clearly that hydrochloric acid increased iron absorption two to threefold. Conversely if pepsin and HCl were administered simultaneously absorption decreased to about 50% of those values. Combined administration of hydrochloric acid and oral iron might therefore be recommended in the treatment of iron deficiency anaemia in gastrectomized patients.

Résumé

L'importance du manque d'acide chloridrique a été étudiée chez des patients de sexe mâle, ayant subi une gastrectomie partielle et ne souffrant pas d'anémie, en déterminant l'activité du plasma après la résorption de fer radiomarqué (citrate de Fe^{59}). La comparaison des résultats avec ceux obtenus chez des sujets sains démontre clairement que l'acide chloridrique augmente la résorption du fer de deux à trois fois. Si par contre l'acide chloridrique est administré en même temps que de la pepsine, la résorption diminue d'environ 50%. L'administration combinée d'acide chloridrique et de fer par voie orale est pour cela préconisée dans le traitement de l'anémie sidéroprive chez les patients ayant subi une gastrectomie.

Zusammenfassung

Bei partiell gastrektomierten männlichen Patienten ohne Anämie wurde die Bedeutung des Salzsäuremangels untersucht durch Bestimmung der Plasmaaktivität nach Resorption von markiertem Eisen (Fe^{59}-Zitrat). Der Vergleich mit den Resultaten bei gesunden Männern ergab, daß Salzsäure die Eisenresorption auf das Zwei- bis Dreifache steigert. Dagegen sank die Resorption auf ca. 50% dieser Werte, wenn Pepsin und Salzsäure gleichzeitig verabreicht wurden. Bei der Behandlung der Eisenmangelanämie gastrektomierter Patienten ist daher eine kombinierte Verabreichung von Eisen und Salzsäure zu empfehlen.

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Immunoelectrophoretic and Autoradiographic Pattern of Hb Binding Serum Proteins

By Antonio Prioli and Liborio Giuffrè

The property of serum proteins to bind Hb has been known for many years.

Haematin added to serum modifies its absorption spectrum (6) because of the bond between it and the albumin (3) forming methaemalbumin.

Under the conditions illustrated below the addition of Hb to serum determines the formation of methaemalbumin the bond is effected between heme and albumin (in the ratio of two molecules of haeme per one molecule of albumin (22), and according to O'Hagan (14) the globulin would not be part of the final compound. Methaemalbumin was found to be present in serum of newborn babies affected by haemolytic disease (26, 24)

Polonovski and Jayle (16) found that the peroxydase activity of Hb increased if serum was added Afterwards they showed that the phenomenon was due to the bond of Hb with an alpha$_2$-globulin, defined as haptoglobin (Hp). The bond is made between globin and Hp in stechiometric ratio of 1 1 Hp is able also to bind carboxy haemoglobin, methaemoglobin, and cyanhaemoglobin.

During recent years new data have been acquired on the structure and genetic properties of Hp, on the characteristics of the haptoglobin-haemoglobin complex, on the physiological properties of such protein, and on the changes induced by various pathological conditions. The serum level of Hp becomes lower in subjects affected by haemolytic diseases (7 13, 1 11 2, 15, 21 17).

According to Fisher Herman et al. (4) an alpha$_1$-globulin would be able to combine with haematine. The experiments of Allison and Rees (1) gave similar results.

Neale et al. (10) have described a Hb binding beta-globulin, defined by Ytman (12) as "haeme binding beta-protein which presents different properties from the haptoglobin it has lower affinity for Hb than haptoglobin it binds with haeme while haptoglobin binds with globin. Moreover it is able to bind not only with Hb, but also with haematine and its content in the serum becomes lower in cases of haemolytic anaemia.

Wheby et al. (25) reported on protein, which is able to bind myoglobin and haematin in vitro It was not possible to establish whether it belongs to the alpha$_2$- or to the beta-globulins. Under normal conditions this globulin contributes for approximately 10% to the total Hb binding capacity of the serum, but in cases of haemolytic anaemia it may represent the entire Hb-binding capacity of the serum.

From our experience (17–18) it results that in subjects affected by thalassaemia major (since the haptoglobin is absent or decreased) the addition of Hb to the serum determines the formation of methaemalbumin and Hb does not appear to bind with any beta globulin. However the formation of methaemalbumin appeared after electrophoresis on paper of the mixture of serum plus Hb. Methaemalbumin did not appear in immunoelectropherograms developed with the same mixture of serum plus Hb. Identical results have been obtained in subjects with sickle cell thalassaemia disease (19).

According to NYMAN (12) benzidine staining of paper-electropherograms of normal adult sera plus Hb, showed the formation of methaemalbumin, of Hb-Hp complex and peroxidase activity towards beta-fractions. The same staining procedure failed to visualize the Hb-binding beta-globulin in immunoelectropherograms developed with the above mixtures of sera plus Hb. In order to identify this beta-globulin, it appeared interesting to study the Hb-binding serum proteins by carrying out immunoelectrophoresis and autoradiography of mixtures of sera plus Hb and methaemoglobin (MetHb) labelled with ^{51}Cr and with ^{59}Fe. For this purpose we tested sera of normal adult subjects, of normal newborn babies, and also of subjects affected by thalassaemia major.

Methods

Immunoelectrophoresis was performed following the technique described by GRABAR (5) and modified by SCHEIDEGGER (23) using the discontinuous buffer system with pH value of 8.6, according to LAURELL (8

Before staining of the immunoelectropherograms the autoradiogram was developed by placing the immunoelectrophoresis slide directly in contact with an autoradiographic film. The time of incubation varied from 23 to 45 days.

Haemoglobin labelled with ^{51}Cr was obtained by tagging human red blood cells with ^{51}Cr, according to MOLLISON AND VEALL (9

By injecting in the ear vein of rabbit 100 μc of ^{59}Fe and reinjecting 30 μc after week haemoglobin labelled with ^{59}Fe was obtained. A week after the second inoculation blood sample was taken and the red cells were haemolyzed.

The methaemoglobin was prepared by adding 10 mg of potassium ferricyanide to every ml of 5 solution of HbO_2.

Quantitative determination of haemoglobin and methaemoglobin labelled with ^{51}Cr and with ^{59}Fe has been made by DRABKIN method and the quantity added to sera was 250 mg per 100 ml of serum.

The sera under examination were taken from fasting subjects and when not used immediately after they were kept at −20 °C.

Rabbit anti-human immune serum was obtained according to PROOM technique (20

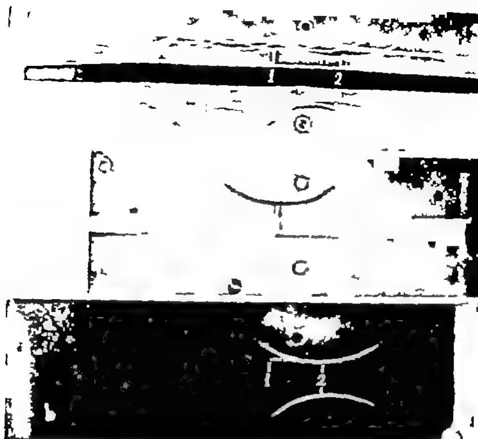


Fig 1 serum of normal adult subject. b serum of normal newborn baby. HbO_2-^{51}Cr has been added to the sera at the concentration of mg 250/100 ml of serum. Top Immunoelectrophoretic pattern. Centre Benzidine hydrogen peroxide staining Bottom Autoradiographic film. The lines indicate 1-haptoglobin 2-beta$_1$-transferrin.

The anti-beta$_1$-transferrin serum was obtained by the Behringwerke. After developing the autoradiograms, the immunoelectrophoretic slides were stained with benzidine. The mixtures of serum plus HbO_2 and MetHb were prepared approximately 15 minutes before the electrophoresis was carried out.

As control, mixtures of the same sera plus ^{51}Cr were submitted to immunoelectrophoresis and autoradiography

Results

Fig 1 top shows the immunoelectrophoretic pattern of the serum of an adult normal subject (a) and of the serum of a newborn baby b HbO_2 labelled with ^{51}Cr was added to the sera, at the concentration of 250 mg/100 ml serum. Benzidine staining (cen-

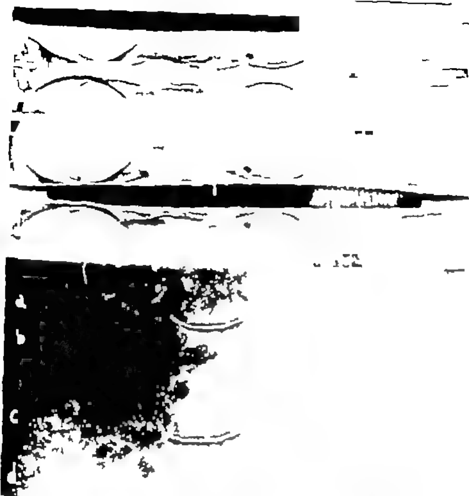


Fig [illegible] Top Immunoelectrophoretic plates after staining with benzidine (photographed with orthocromatic material) normal adult serum plus HbO_2 ^{51}F ('a') and plus MetHb-^{51}F ('c'); Serum of subject with thalassemia major plus HbO ^{51}F ('b') and plus MetHb-^{51}F ('d') The lines indicate haptoglobin Bottom Autoradiographic film of the same mixtures of sera plus HbO_2 ^{51}F and MetHb-^{51}F

tre of fig 1) shows that only the normal adult serum contains haptoglobin. After developing the autoradiography it appears that (bottom) the ^{51}Cr activity is present in the haptoglobin and in a beta-globulin of the normal adult serum, and only in a beta-globulin of the serum of the newborn baby

Under the present experimental conditions, the Hb-beta globulin complex does not react with benzidine and hydrogen

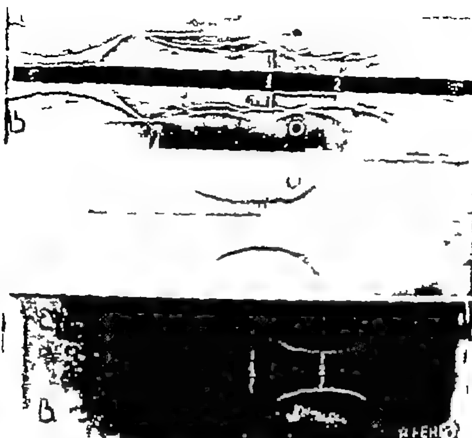


Fig 3. Mixtures of normal adult sera plus MetHb ^{51}Cr (a and b). The immune serum is lacking antigamma globulin antibodies. Top Immunoelectrophoretic pattern Centre benzidine hydrogen peroxide staining Bottom autoradiographic film. The lines indicate 1 haptoglobin 2 haem-transferrin.

peroxide Moreover methaemalbumin does not appear in the immunoelectropherograms developed with serum plus excess of Hb. The migration pattern of the same mixtures on paper electrophoresis clearly indicated the presence of methaemalbumin.

Immunoelectropherograms of normal adult serum and of serum of subject with thalassaemia major plus HbO_2-^{59}Fe and Met Hb ^{59}Fe are shown in fig 2 It appears that haptoglobin is absent in the serum of the thalassaemic subject and that the ^{59}Fe activity is present only in the haptoglobin The immunoelectrophoretic and autoradiographic pattern of normal sera plus MetHb ^{51}Cr is identical to that obtained by adding HbO_2 ^{51}Cr to the same sera (fig 3

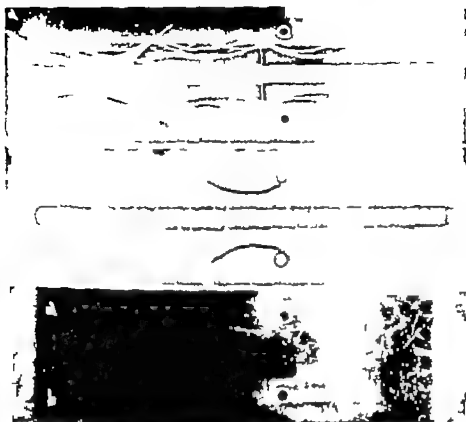


Fig 4 Serum of subject with thalassaemia major splenectomized two years ago plus HbO_2 ^{51}Cr and plus MetHb ^{51}Cr Top Immunoelectrophoretic pattern Centre after staining with benzidine; Bottom autoradiographic film.

Fig 4 shows the pattern of the serum of a subject with thalassaemia major splenectomized two years ago after addition of HbO ^{51}Cr and MetHb-^{51}Cr Both benzidine staining and autoradiography indicate that the added HbO_2 and MetHb are present in the haptoglobin, and that the evidence of such compounds in the albumin and in the beta-globulins failed.

According to the immunoelectrophoretic characteristics of the beta-globulin showing Hb binding properties, the identification of such a beta-globulin has been performed by developing immunoelectrophoresis of normal adult serum and of the serum of the thalassaemic subject with anti-beta[1] transferrin serum (fig 5) HbO_2 ^{51}Cr has been added to the sera It results that the beta-globulin



Fig 5 normal adult serum plus HbO ^{51}Cr b serum of subject with thalassaemia major plus HbO_2 ^{51}Cr Immunoelectrophoresis has been developed with anti-beta$_1$ transferrin serum. Top Immunoelectrophoretic pattern. Bottom Autoradiographic film.

showing Hb binding properties is beta$_1$ transferrin, that the beta$_1$ transferrin of the serum of the thalassaemic subject is not able to bind the added HbO_2 ^{51}Cr and that this complex (Hb-beta$_1$ transferrin) does not react with benzidine and hydrogen peroxide.

Discussion

The present investigation shows that the Hb-binding proteins of normal adult sera are haptoglobin and beta$_1$ transferrin, as obtained by immunoelectrophoretic and autoradiographic techniques. Haptoglobin is able to bind HbO_2 or MetHb in the same way and it prevents the dissociation in vitro of HbO_2 or MetHb. In experiments carried out with the above mentioned compounds labelled with ^{59}Fe, the Fe^{59} activity is discoverable in the haptoglobin, even if the immunoelectropherogram is carried out some time after the incubation of the mixture of serum and Hb ^{59}Fe or MetHb ^{59}Fe has been made.

The presence of appreciable quantities of haptoglobin in the serum of splenectomized subjects with thalassaemia major agrees with results of our previous experience.

The addition of excess of HbO_2 or MetHb to the serum determines the formation of a complex between such compounds and $beta_1$-transferrin, as results from the immunoelectropherograms developed with anti-beta[1] transferrin serum. The Hb-binding capacity of $beta_1$-transferrin seems however to be limited. Transferrin contained in the serum of subjects with thalassaemia major is not able to bind Hb. This is probably due to a nearly complete saturation.

As regards the findings of POLONOVSKI (16), NYMAN (12), NEALE (10), O'HAGAN (14) and others, our results present some common aspects and differ in others. NEALE (10) in 1958 referred that a not identified beta-globulin is able to bind haemoglobin. NYMAN (12) in 1960 confirmed such a finding and specified that the Hb binding beta-globulin has the same mobility on paper electrophoresis as beta transferrin and that the globin is not present in the complex formed by Hb or its derivatives and the beta-globulin.

On the basis of our results it seems that the bond between HbO_2 or MetHb and $beta_1$ transferrin is effected by the globin. In the experiments carried out with HbO_2 or MetHb labelled with ^{51}Cr that labels the globin, the autoradiograms show the ^{51}Cr activity in the beta-globulin, while by using ^{59}Fe as a label, no activity was found in the beta transferrin.

Moreover the HbO_2 or MetHb-beta transferrin complex has no peroxydase activity. Further investigations should be carried out on the characteristics and properties of this complex. The lack of methaemalbumin in the immunoelectropherograms in spite of the excess of HbO_2 and MetHb added to the sera, is not explained.

In an earlier paper on immunoelectrophoretic pattern of sera of subjects with thalassaemia major and with sickle-cell-thalassaemia disease, we obtained the same results and we referred that this phenomenon is probably due to the non identical antigenic property of methaemalbumin and of albumin. By washing the slides, methaemalbumin could be cleared out and the benzidine stain performed afterwards failed to demonstrate peroxydase activity in the albumin.

Summary

Excess of HbO_2 or MetHb labelled with ^{51}Cr and with ^{59}F has been added to sera of normal adults, of newborn babies, and of subjects with thalassaemia major

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Verhalten der H^3 Thymidin Inkorporation im fraktioniert strahlengeschädigten Knochenmark*

Von B. Choné

Der durch bestimmte Radioisotope faßbare und im Autoradiogramm lokalisierbare Zellumsatz proliferativer Gewebe gab Veranlassung, die Funktionsdynamik der Knochenmarkzellen im Rahmen der Strahlentherapie zu überprüfen. Zu diesem Zweck wurden zu verschiedenen Zeitpunkten entnommene Knochenmarkpunktate in vitro mit H^3 Thymidin (2 μC in 0,4 ml NaCl EDTA-Lösung spez. Akt. 1,9 C/mM) inkubiert und der Markierungsindex in bestrahlten und unbestrahlten Knochenmarkabschnitten bestimmt (Stripping Filmmethode).

Material und Methodik

Es handelt sich um Querschnittsuntersuchungen an nahezu 150 Karzinomkranken, die durchweg mit einer lokalen Tumordosis von 5000–6000 R Co^{60} bestrahlt wurden, was bei entsprechender Feldlage eine Mitbelastung des Knochenmarkes bis 4000–5000 R zur Folge hatte. Die effektive Einzeldosis lag zwischen 200–300 R, das Bestrahlungsintervall betrug in der Regel 24 Stunden. In eingehenden Voruntersuchungen war der pathomorphologische Ablauf der lokalen Knochenmarkschädigung unter gleichen Bestrahlungsbedingungen analysiert worden (4, 5).

Resultate

Wie bereits beim morphologischen Aspekt der hämatologischen Strahlenschädigung zum Ausdruck kam, betrifft der initiale Zellverlust am stärksten die Erythropoese, deren Zellumsatz normalerweise etwa doppelt so rasch erfolgt, wie der der Myelopoese. Die daraus bei hoher einzeitiger oder fraktionierter Strahlenbelastung resultierende *erythropoetische Insuffizienz* ist angesichts der

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In Anlehnung an Vortrag am X. Int. Haematologen-Kongreß Stockholm, 1964.

Die Untersuchungen wurden mit Unterstützung des Bundesministeriums für Wissenschaftliche Forschung durchgeführt.



Abb. 1 Normaler Knochenmarkausstrich, H³T in vitro inkubiert. Stripping Filmautoradiographie + HE-Färbung Expositionszeit = 10 Tage M 256 : 1 Lebhafte Markierung zahlreicher roter und weißer Entwicklungsstufen.

Abb. 2 Markierter Promegakaryocyt, 15 Min. nach Knochenmark-Erstexposition mit 300 Erythropoese aktiv. Stripping-Filmautoradiographie – HE-Färbung Expositionszeit = [illegible] Tage M 256 : 1



Abb. 3. Knochenmarkausstrich, 90 Min. nach Erstexposition mit 250 r effektiver Knochenmarkdosis (Co^{60}). Erythropoetischer Reifungszylinder unmarkiert. Links: Bildmitte diskret markierte Retikulum-Zelle, rechts starker markierter Metamyelozyt. M 256 : 1

langen Erythrozyten-Lebensdauer peripher «maskiert». Jedoch tritt die Schädigung des Funktionsstoffwechsels innerhalb der Erythropoese mittels H³-Thymidin-Inkubation sehr frühzeitig und prägnant in Erscheinung (Abb. 1–5).

Die Markierungstendenz der unreifen Erythroblasten läßt schon nach 1–2 Teilbestrahlungen im genannten Dosisbereich sprunghaft nach, obwohl zu diesem Zeitpunkt noch reichlich Vorstufen vorhanden sind (Abb. 2 und 3). Eine vorübergehende Thymidin-Einbaustörung scheint schon wenige Minuten nach dem ersten Strahleninsult möglich zu sein (Abb. 3). Oft ist zu diesem Zeitpunkt in einem ausstrichmäßig erfaßten Erythron kaum eine Zelle markiert. Diese Beobachtung spricht zweifellos für eine hohe Radiosensibilität der Erythroblastengeneration. Diese mag relativ sein, wenn man die kürzere Reifezeit und die stärkere Proliferationsaktivität der Erythropoese berücksichtigt, fällt aber gerade deshalb um so mehr ins Auge.

Die *myelopoetische Entwicklungsreihe* verhält sich unter den gegebenen Bestrahlungsbedingungen (24 Std. Intervall) offensichtlich strahlenresistenter. Im Gegensatz zu der mit 3–4 Tagen begrenzten



Abb. 4. 3 Proerythroblasten, li. Mitosestadium (Metaphase), mittlere Zelle in DNS-Synthese (H^3-T markiert), rechts Ruheform. Knochenmarkautoradiographie, 20 Stunden nach Erstexposition mit 250 r. Links unten Normoblast.

Abb. 5. Markierter binucleärer Makroblast, 48 Stunden nach Erst- und 24 Stunden nach Zweitexposition des Knochenmarkes mit je 250 r. M 403:1

Abb. 6. Markierter Myelozyt mit eigenartigen strahleninduzierten Knospenbildungen. Fraktionierte Knochenmarkbelastung — 900 r Co^{60}

Lebensdauer der Granulozyten verläuft die Zellausreifung wesentlich langsamer und der strahleninduzierte quantitative Zellverlust im Knochenmark tritt dadurch protrahierter in Erscheinung. Von den morphologisch intakten Zellen ist ein Großteil markiert, und selbst manifest geschädigte Zellelemente scheinen noch die Fähig-



Abb. 7 Pathologischer Myelozyt (Rosettenform) deutlich H³ T-markiert neben ähnlicher unmarkierter Zellform. Zustand nach 1200 r Knochenmarkdosis (4 Bestrahlungen).

Abb. 8. Partiell markierter Promegakaryozyt. Knochenmarkautoradiographie nach 3. fraktionierter Strahlenbelastung mit lokaler Knochenmarkdosis von 600 Bestrahlungsintervall = 24 Stunden. M 450:1

keit zur Proliferation zu benützen (Abb 6 und 7) Ob diese Potenz ausreicht, auch noch weitere Zellfunktionen auszuüben oder ob sie sich in einer endomitotischen Ausreifung der Zellen erschöpft, muß freilich offen bleiben. In Abbildung 8 ist eine eigenartige Teilmarkierungsform bei einem Promegakaryozyten festgehalten.

Das normalerweise nur wenig und erst bei »Reizzuständen stärker hervortretende Knochenmarkreticulum erweist sich in funktioneller Hinsicht als besonders strahlenresistent. Es toleriert eine fraktionierte Strahlendosis von 2000–3000 R Co⁶⁰ ohne Verlust seiner proliferativen Leistungsreserve und bildet in diesem Zusammenhang zugleich den Ausgangspunkt für die postradiologische Knochenmarkregeneration (Abb 9 und 10)

Im Stadium der Knochenmarkregeneration pflegt übrigens die Erythropoese der myelopoetischen Restitution voranzugeben, was im Vergleich zum primären Verhalten der roten Zellreihe während der Strahlenexposition bemerkenswert erscheint (6)



Abb. 9. Knochenmark-Autoradiographie nach 2000 r Co⁶⁰ Dosis. Stadium der beginnenden «Reticulose» mit lebhaftem H^3-T-Einbau in große Reticulumzellen. Rote und weiße Entwicklungsreihe stark zurückgedrängt (M 256:1)

Diskussion

Das bei fraktionierter Strahlenbelastung zu beobachtende funktionelle Verhalten der Knochenmarkzellen weist gegenüber der rein morphologischen Perspektive einige Besonderheiten auf, die in autoradiographischen Paralleluntersuchungen charakterisiert werden können. Das zu diesem Zweck verwandte H^3-Thymidin ist auf Grund seiner «Affinität» zum Zellstoffwechsel und wegen seiner günstigen Strahlengeometrie als Markierungssubstanz optimal geeignet.

Die Auswirkungen einer ionisierenden Strahlung auf das hämatopoetische System hängen in entscheidender Weise von der Dosishöhe, der Bestrahlungsform, dem bei fraktionierter Strahlenbelastung gegebenen Zeitintervall und den jeweiligen strahlenbiologischen Gegebenheiten ab, wozu Strahlenqualität und individuelle Faktoren gehören.

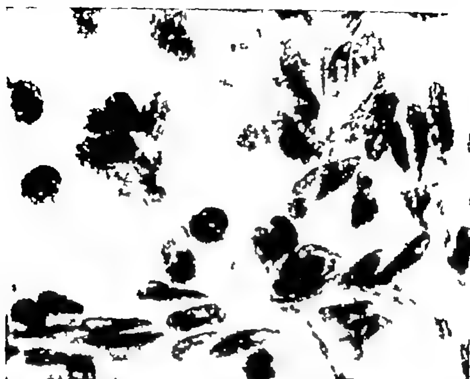


Abb. 10. Weitgehend komplette reticulo-plasmacelluläre Transformation mit noch zum Teil kräftiger H^3 T Inkorporation. Im unteren Bildanteil Kapillar Endothelmember. Fraktionierte Knochenmarksdosis = 3000

Für die unterschiedliche Strahlenreaktion von erythro- und myelopoetischer Reihe dürfte neben der Abweichung in der Proliferationsdynamik die unterschiedliche Reifungszeit maßgebend sein, die innerhalb der roten Zellreihe beschleunigter abläuft. Diese Tatsache in Verbindung mit der therapeutisch üblichen Fraktionierung und dadurch bedingten Strahlenkumulation führt zwangsläufig zu einer frühzeitigeren Beeinträchtigung der Erythropoese deren Proliferationsaktivität andererseits in der Knochenmarkregenerationsphase führend in Erscheinung tritt. In diesem Stadium gewinnen auch die radiobiologischen Begleitumstände an Bedeutung wie sich aus vergleichenden Untersuchungen zwischen konventioneller Röntgentiefen- und Co^{60}-Supervolttherapie ergeben hat (3 6).

ROSENTHAL et al. (12) die den hämatologischen Schadensablauf am Rattenknochenmark nach 700 r Ganzkörperbestrahlung

untersuchten, konnten die bereits von DENSTAD (8) und BLOOM (1) erfaßte frühzeitige «Verdrängung» des erythropoetischen Systems bestätigen und gleichzeitig eine absolute Vermehrung der Plasma-Retikulumzellen nachweisen. Die Erythro- und Reticulozytenzahl erwies sich bis 4 Stunden post rad. signifikant erhöht, was einem beschleunigten Reifeprozeß der poly- und orthochromatischen Erythroblasten zu Reticulozyten zugeschrieben wird. Dagegen ist die Radioeiseninkorporation in das Knochenmark nach HENNESSY UND HUFF (11) innerhalb von 24 Stunden schon nach [illegible] r Ganzkörperbestrahlung um 90 % reduziert. Bei der 12 Tage nach dem Strahleninsult beginnenden Regeneration traten zuerst erythropoetische Zellinseln in Erscheinung, Myelo- und Megakaryopoese folgten mit einiger Verzögerung nach.

In vergleichenden Untersuchungen zur Radiosensibilität des myelo- und erythropoetischen Gewebes kamen VALENTIN UND PEARCE (14) zu dem Schluß, daß die weiße Zellreihe eine geringere funktionelle Zellreserve habe, die sich einmal in der prompten Leukopenie nach Bestrahlung dokumentiere, andererseits auch für den späteren Zeitpunkt der Knochenmarkregeneration verantwortlich zu machen sei.

Von diesem Gesichtspunkt aus muß man der Myelopoese zweifellos eine größere «Anfälligkeit» zugestehen. Jedoch hat diese Feststellung keine Beziehung zum eigentlichen Begriff der Radiosensibilität, da mit ihr bereits ein Folgezustand charakterisiert und der Primäreffekt an der Einzelzelle ignoriert wird (7).

Die Durchsicht der einschlägigen Literatur zeigt, daß systematische Knochenmarkuntersuchungen am Menschen unter den Bedingungen der Supervolttherapie bisher fehlen. Dies betrifft sowohl zytomorphologische Studien als auch funktionelle Untersuchungen mit Radioisotopen. In letzter Zeit ist lediglich eine Arbeit von GOSWITZ et al. (10) erschienen, die über zelluläre Bestrahlungseffekte bei lokaler Co^{60}-Bestrahlung an [illegible] Tumorpatienten berichtet. Die Knochenmarkpunktionen fanden hier – analog eigenem Untersuchungsregime – vor, während und nach Durchführung der Strahlenbehandlung statt. Die Auswertung erfolgte durch quantitative Bestimmung von je 1000 kernhaltigen Zelltypen, zusätzlich wurde das Knochenmark mit H^3-Thymidin *in vitro* inkubiert. Die verabfolgten Tumordosen schwankten zwischen 4000–6000 r. Der Grad der lokalen Knochenmarkveränderungen (Hypoplasie) erwies sich als dosisabhängig, war jedoch nicht ausreichend, um eine spätere

Regenerationsleistung des Knochenmarkes im Bestrahlungsfeld zu verhindern. Die erwachende Markfunktion machte sich durch eine lebhafte H³-Thymidin-Markierung der neugebildeten Knochenmarkzellen bemerkbar, die teilweise schon erstaunlich frühzeitig nach Bestrahlungsabschluß einsetzte, was mit dem inhomogenen Treffer-Prinzip der ionisierenden Strahlung zusammenhängen dürfte.

Zusammenfassung

Vergleichende Knochenmarkuntersuchungen an mit H³-Thymidin markierten Ausstrichpräparaten unter den Bedingungen einer fraktionierten Teilkörperbestrahlung ergaben charakteristische Unterschiede im Markierungsmodus zwischen Erythro- und Myelopoese. Die aus einer durchschnittlichen Einzeldosis von 200–300 r effektiver Knochenmarkbelastung resultierende funktionelle Zellbeeinträchtigung betrifft in erster Linie die Erythropoese, wofür vermutlich neben der durch die höhere Proliferationsaktivität bedingten Steigerung der Radiosensibilität das Bestrahlungsintervall maßgebend sein dürfte. Die von den Retikulumzellen repräsentierte, undifferenzierte Knochenmark-Matrix weist bezüglich ihrer funktionellen Leistungsreserve eine hohe Strahlenresistenz auf.

Summary

Comparative study of bone marrow preparations labelled with H³-thymidine after fractionated partial irradiation showed characteristic differences in marking between erythropoiesis and myelopoiesis. The functional cellular impairment resulting from an average single effective dose of 200 to 300 r to the marrow affects erythropoiesis primarily; this is probably due, apart from the increased radiosensitivity on account of the high rate of proliferation, to the particular radiation interval. The undifferentiated matrix represented by reticulum cells is highly resistant to radiation in its reserve of functional capacity.

Résumé

Des études comparées de la moelle osseuse ont été faites sur des frottis radiomarqués à la thymidine tritiée. Ces frottis de moelle obtenus après une radiothérapie fractionnée montrèrent des différences caractéristiques entre le marquage de l'érythropoïèse et de la myélopoïèse. L'inhibition fonctionnelle, résultant de l'application isolée d'une dose moyenne de 200–300 r sur la moelle, touche en premier lieu l'érythropoïèse. Il est probable que l'intervalle des irradiations ainsi que la radiosensibilité plus grande de l'érythropoïèse due à sa prolifération plus intense, en soit surtout responsable. Les cellules-souches sont, quant à leur réserve fonctionnelle, très résistantes à l'irradiation.

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Red Cell Acid Phosphatase in Normal and Glucose-6-Phosphate Dehydrogenase Deficient Sardinian Subjects*

By Francesco Schettini, Tullio Meloni, Clara Mela and Giovanni Fanciulli

It has been recently demonstrated that the acid phosphatase activity of red cells is related to the red cell acid phosphatase phenotype detected by starch gel electrophoresis (1, 2, 3) and identified as A, BA, B, CA and CB. Considerable variations in the level of activity are present between individual of any given type (2). This relationship between the levels of red cells acid phosphatase activity and red cell acid phosphatase phenotype is very important in the evaluation of the activity of this enzyme in two or more groups of subjects.

We have studied the activity of red cell acid phosphatase in Sardinian subjects with erythrocyte glucose-6-phosphate dehydrogenase deficiency in relation to the phenotype.

Materials and Methods

We have examined 52 normal subjects (aged from 2 to 33 years), 8 enzyme deficient subjects (males and females), 17 children, that have suffered of haemolysis by vicia faba four months ago, and 17 intermediate females. The investigation was carried out only in Sardinian subjects.

Fresh blood obtained by venipuncture is collected with ACD solutions as anticoagulant and assayed as soon possible.

Red cells are washed three times in 0.15 M Na Cl, centrifuging for 5 minutes at 3000 rpm at +4 °C. Buffy-coats are discarded.

The *assay of glucose-6-phosphate dehydrogenase (G6PDH) activity* of erythrocytes is made according to the method of Kirkman and Riley (4). The results are expressed as units per 100 ml of red blood cells.

The *assay of acid phosphatase activity* of erythrocytes is performed with p-nitrophenyl-phosphate as substrate (5, 1).

Haemolysates are prepared by addition of 9 volumes of distilled water to one volume of washed packed red cells.

The substrate solution is made fresh daily with disodium p-nitrophenyl-phosphate

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(SIGMA 104 substrate) 0.02 M in 0.05 M citrate buffer pH 6.0. The pH is adjusted to 6.0 with 0.05 M citric acid.

The assay is carried out in centrifuge tubes for each sample 3 tubes are prepared, each containing 0.50 ml of substrate solution and 0.40 ml of distillated water The reaction is started by addition of 0.10 ml of haemolysate. In the first tube the reaction has stopped immediately by the addition of 2.0 ml of trichloracetic acid 10 g% (TCA). The remaining tubes are incubated in a water bath at 37 °C for 15 and 30 minutes before 2.0 ml of TCA is added.

The tubes are centrifuged down at 3000 rpm per 5 minutes. Aliquots of 1.0 ml of the clean supernatant are transferred to clean tubes and the p-nitrophenol (PNP) color is developed in the assay and in the standard tubes by the addition of 4.0 ml of 0.5 M NaOH. The intensity of color is measured at 415 μm (Spectrophotometer Beckman DU) against blank of 1.0 ml of distillated water and 4.0 ml of 0.05 N NaOH.

The quantity of p-nitrophenol liberated in 30 minutes is estimated by means of extinction coefficient of p-nitrophenol (17.56 at 415 μm)

Determinations of haemoglobin in haemolysate are carried out using 0.10 ml of the assay haemolysate and 4.90 ml of Drabkin solution. The resulting color is read against blank of Drabkin's solution at 540 μm.

The red cell acid phosphatase activity is expressed as micromoles of p-nitrophenol liberated in 30 minutes at 37 °C per g of haemoglobin.

Electrophoretic studies of red cell acid phosphatase phenotypes are carried out by the method of Hopkinson et al. (1).

Haemolysates are prepared by addition of 2 volumes of distillated water to one volume of washed packed red cells.

Red cell haemolysates are subjected to electrophoresis in starch gel at pH 6.0. The gel (Starch of Connaught Laboratories) is made according the reccomendation by the makers, using succinic acid/Tris buffer (0.0025 M in succinic acid, 0.0046 M in Tris, pH 6.0). A citric acid/NaOH buffer (0.41 M in citric acid, pH 6.0) is used as bridge solution. The haemolysates are inserted into the gel on very tick filter paper (Whatman n. 17) with strips of 1.30 cm × 0.50 cm. Electrophoresis is carried out horizontally at 6 volts/cm for 16–17 hours at +4 °C. The gels are then sliced horizontally and placed in plastic box. Fifty ml of 0.005 M phenolphtaleine diphosphate disodium (SIGMA) in 0.05 M citrate buffer pH 6.0 are poured on the cut surface of the gel. Incubation is performed at 37 °C for 3 hours then the mixture is poured off and about [illegible] ml concentrated ammonia is added. The ammoniacal atmosphere makes the surface of the gel alkaline and the areas where the phenolphtalein has been liberated by acid phosphatase are revealed as red zones.

The identification of phenotype must be made as soon possible because sharp definition persists only for about 30–60 minutes.

Results

Subjects with red cell acid phosphatase phenotype B (table I) In 52 subjects with normal glucose-6-phosphate dehydrogenase activity we have observed a mean value of red cell acid phosphatase activity of 163.6 μmoles of PNP per g of haemoglobin, liberated in 30 minutes at 37 C with a variance (S^2) of 1124.5 and a standard deviation(s) of ± 33.52.

In 17 subjects with deficiency of G6PDH we have observed a mean value of acid phosphatase activity of 156.5 μmoles/g of

Table I
Subjects with red cell acid phosphatase phenotype B.

Normal			G 6 PDH deficient			G 6 PDH intermediate females		
No.	Acid [illegible]	G 6 PDH [illegible]	No.	Acid [illegible]	G 6 PDH [illegible]	No.	Acid [illegible]	G 6 PDH [illegible]
1	126.4	201	1	160.3	0	1	146.7	[illegible]
2	153.3	292	2	151.2	0	2	153.8	108
3	116.5	211	3	127.5	0	3	153.2	95
4	164.8	333	4	212.0	18.4	4	141.1	83
5	206.6	182	5	212.0	18.4	5	110.6	79
6	159.1	118	6	117.4	0	6	126.2	54
7	222.4	140	7	83.8	11	7	188.5	258
8	195.3	401	8	152.4	0	8	162.5	111
9	141.8	124	9	256.2	0	9	157.7	[illegible]
10	138.8	120	10	212.2	18.4	10	156.8	221
11	233.9	332	11	162.9	13.4	11	190.8	122
12	110.6	291	12	124.2	0	12	151.2	[illegible]
13	174.9	147	13	134.3	9.6	13	128.7	156
14	133.5	170	14	133.4	4.56			
15	165.9	182	15	141.1	0			
16	155.8	214	16	138.8	3.8			
17	170.4	191	17	117.4	0			
18	135.4	217						
19	172.7	165						
20	152.4	300						
21	123.0	214						
[illegible]	109.5	200						
23	232.5	201						
24	174.9	186						
25	147.9	198						
26	186.2	317						
27	153.5	197						
[illegible]	123.0	166						
29	258.5	461						
30	172.7	325						
31	155.8	241						
32	149.0	257						

haemoglobin (S^2 = 1871.7 s = ±42.25). In 13 intermediate females, mothers of enzyme deficient males, we have showed a mean value of acid phosphatase activity of 148.4 µmoles/g of haemoglobin (S^2 = 396.2 s = ±19.90).

The difference between normal and G6PDH deficient erythrocytes is not significant (t = 0.509 and respectively t = 1.766 P = 0.05) (table II).

Table II

Statistical analysis.

	Mean value µmol./g Hb	Variance	Standard deviation	calculated F=0.05
Subjects with red cell acid phosphatase phenotype B				
Normal subjects	163.6	1124.5	±33.52	–
G6PDH deficient subjects	136.5	1871.7	±42.25	0.509
G6PDH intermediate females	148.4	396.2	±19.90	1.766
Subjects with red cell acid phosphatase phenotype BA				
Normal subjects	147.0	803.7	±28.34	
G6PDH deficient subjects	138.9	1575.2	±39.68	0.609
G6PDH intermediate females	174 4	624.9	+24.98	2.091

Table III

Subjects with red cell acid phosphatase phenotype BA.

Normal			G6PDH deficient			G6PDH intermediate females		
№	Acid phosphatase activity µmol./g Hb	G6PDH units/100 ml of RBC	№	Acid phosphatase activity µmol./g Hb	G6PDH units/100 ml of RBC	№	Acid phosphatase activity µmol./g Hb	G6PDH units/100 ml of RBC
1	125.8	301	1	83.7	12	1	145.6	216
2	143.9	341	2	98.4	26	2	208.6	107
3	151.6	240	3	110.9	15	3	171.6	41.8
4	144.5	160	4	144.5	0	4	173.8	67 1
5	169.3	207	5	151.2	9.5			
6	145.6	148	6	225.8	15.9			
7	220.1	255	7	143.3	17.6			
8	165.9	156	8	155.5	8.34			
9	147.8	196						
10	102.7	206						
11	128.7	186						
12	143.6	203						
13	123.0	211						
14	128.7	225						
15	112.9	178						
16	186.2	155						
17	123.0	161						
18	126.4	193						
19	158.8	182						
20	209.9	249						

Subjects with red cell the acid phosphatase phenotype BA (table III)
In 20 subjects with normal G6PDH activity we have observed a mean value of red cell acid phosphatase activity of 147 ▮ µmoles/g of haemoglobin (S^2 = 803 7 s = ±28.34)

In 8 subjects with deficit of G6PDH we have observed a mean value of acid phosphatase activity of 138.9 µmoles/g of Hb (S^2 = 1575.2 s = ±39 68) In 4 intermediate females we have observed a mean value of acid phosphatase activity of 174 4 µmoles/g of Hb (S^2 = 624 9 s = ±24.98)

The difference between normal and G6PDH deficient erythrocytes is not significant (t = 0 609 and respectively t = 2.091 P = 0 05) (table II)

Discussion

Several enzymatic defects have been showed in the erythrocytes of subjects with favism. The defect of G6PDH activity is the most common and determines an important change of erythrocyte metabolism. The reduction of glutathion-synthetase activity is another abnormality (6) A reduction of catalase activity has been demonstrated in Negroes with deficiency of G6PDH (7) and in Sardinian subjects with favism (8) The pyrophosphatase activity in G6PDH deficient subjects is generally decreased and in initial phase of acute haemolytic crisis presents a further critical diminution (9 10)

The reduction of red blood cell acid phosphomonoesterase (acid phosphatase) has been showed in Caucasian subjects with G6PDH deficiency however the activity of acid phosphomonoesterase has been demonstrated within the normal range in Negro males with G6PDH deficiency (11 12) This finding appears to be a consistent biochemical difference between the two racial groups (11 12) A deficiency of acid phosphomonoesterase has been also demonstrated in Greek subjects with favism and in their mothers (13)

The demonstration of existence of several phenotypes of red cell acid phosphatase and the correlation between the phenotype and the level of enzymatic activity have suggested new studies of the acid phosphatase activity in subjects with G6PDH deficiency However it is necessary (1) to detect the acid phosphatase phenotypes comparing groups of subjects because considerable variations of enzyme activity are present between each phenotype.

Our researches have been therefore made in Sardinian subjects of corresponding phenotype acid phosphatase. We have not demonstrated any difference of activity of red cell acid phosphatase between normal subjects and subjects sensitive to fava beans and in their mothers with intermediate values of enzyme activity

The enzymatic primary abnormality of the erythrocytes in Caucasian subjects with favism is, however the deficit of glucose 6-phosphate dehydrogenase. This enzyme has a particular biochemical characteristics such as to definite Mediterranean variant of G6PDH' (14) Other enzymatic abnormality are incostant and are difficult to show exactly

Summary

The activity of red cell acid phosphatase has been investigated in Sardinian subjects with glucose-6-phosphate dehydrogenase deficiency in relation to the acid phosphatase phenotype. No difference of red cell acid phosphatase activity has been observed between normal subjects and subjects sensitive to fava beans and their mothers.

Résumé

L activité de la phosphatase acide des érythrocytes été étudiée chez des sujets sardaignais ayant une déficience en déhydrogénase du glucose-6-phosphate et mise en relation avec le phénotype de la phosphatase acide. Aucune différence de l'activité de la phosphatase acide des érythrocytes ne fut constatée entre sujets normaux et sujets souffrant de favisme ainsi que de leurs mères.

Zusammenfassung

Bei Sardiniern mit einem Mangel an Glukose-6-phosphat-Dehydrogenase wurde die Aktivität der sauren Erythrozytenphosphatase untersucht und zum Phänotypus der sauren Phosphatase in Beziehung gesetzt. Es ergab sich keine Differenz der Aktivität zwischen Gesunden einerseits und Patienten mit Favismus und deren Müttern anderer seits.

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A Major Pitfall in the Use of the Same Radioisotope for the Repetition of Erythrocyte and Other Survival Studies*

By Mortimer Lorber

Many investigators have performed serial determinations of erythrocyte life span using a single isotope. Ideally in such cases, sufficient time elapses so that all the initially labeled cells disappear from the circulation before the repeat procedure is begun. Thus, each survival study is independent and the usual calculation methods are correct. However in some reports (1—8) the interval was less. When that is the case, the life span of the initially administered cells is determined as usual. But during the repeat determination two labeled cell populations are present, one given for that procedure and those cells from the original one which persist during the subsequent survival study To distinguish them requires modifications of the routine computation method.

Two modified calculation procedures shall be described. The selection of which to use depends on the fate of the erythrocytes tagged in the initial procedure which continue to circulate following the readministration of the isotope. The two major possibilities are that those cells maintain their original life span independently of those administered for the repeat procedure (Method I) or that whatever occurrence induced the performance of the repeat study affected both circulating cell populations similarly (Method II)

If the correct alternative cannot be selected prior to performing the second isotope study then a different indicator must be employed in the latter so that each population of labeled red cells may be unequivocally distinguished from the other This would always

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be the most accurate method for assessing survival, but it has the disadvantage of requiring additional experience and perhaps equipment to permit the use of another radioisotope. However if the pattern of cell destruction may be correctly presupposed based on knowledge of comparable clinical or experimental situations in which the usual single survival procedure per subject had been performed, then selection of one of the calculation methods presently reported should yield acceptable data.

Calculation Methods

Hypothetical radiochromium red cell survival data are listed in tables I and II as that isotope is most commonly used to determine erythrocyte life span. However the computation methods are applicable to other labels as well, e. g. DFP[32] C[14]-glycine, etc. For simplicity the problem of elution (9—11) has been disregarded.

General Comments. For brevity only the zero isotope value and the values for days 1 5 and 20 of the repeat determination are tabulated. The data are based on an arbitrary selection of the listed hematocrit values, as well as of 1448 cpm/ml as the extrapolation to zero time for the newly labeled cells of the repeat survival study and 52 cpm/ml as the baseline counts emanating from those previously labeled cells present in the zero sample of the latter procedure. All other values have been derived by semilogarithmic plotting (12) t the various half-times noted in each table and calculated according to the indicated methods.

In both procedures (tables I and II) the net counts after subtraction of the room background are recorded in column 1 and the hematocrit values in column 2. The former figures are corrected to what they would be at hematocrit of 50% by ratio (col. 1 : x = col. 2 50) The adjusted values appear in column 3. This is preferable to listing them as counts per minute per milliliter of packed red cells (13, 14) as the numerical values obtained are more easily used in these calculation methods. The correction process assumes a constant red cell mass (11 13—15) minimizing the effects of day to day differences in hydration and, in animals, splenic tone so that decrease in radioactivity would reflect the subject' hemolytic state and not those extraneous factors. Less commonly as following repeated exposures to or rapid recovery from the effects of hematoxic agent, the hematocrit values might change appreciably during survival study reflecting alterations in the red cell mass. It would then be more accurate not to correct the hematocrit percentages to fixed value (16)

Had the subject had no previous isotopes, as is ordinarily the case when single survival study is performed, the values in column 3 would be plotted semilogarithmically extrapolated to zero time, and the half-time of disappearance of the red cells determined.

This paper though, deals with the situation in which the subject does have previously administered isotope in his erythrocytes at the beginning of the repeat procedure. This zero level does not remain fixed during the followup study but progressively diminishes at a rate reflecting destruction of the labeled cells. This decrease must be considered in modifying the usual calculation procedure.

Method I This should be employed when the rate of destruction of the initially labeled cells is believed to continue unchanged during the repeat study On semilogarithmic graph paper the point on the ordinate representing the isotope content of the zero blood sample of the *repeat* study is connected with its half value on the day corresponding to the half-time of disappearance ascertained in the *initial* procedure (fig. 1) For each sample obtained during the repeat procedure, the corresponding value of the over-

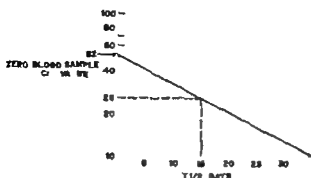


Fig. 1. Progressive diminution of the zero sample isotope level during the repeat determination of erythrocyte life span (calculation method I). The T½ is that of the initial survival study

Table I

Differentiation of isotope counts of red cells labeled in initial and repeat survival studies when the former maintain their original life span.
Calculation Method I.

	1	2	3	4	5
Repeat study samples	Total counts cpm/ml	HCT %	Total counts converted to 50% HCT	Zero blood samples estimated values based on initial survival study*	Repeat study values*
Zero Sample	45	41.7	52		
Day 1	1236	42.5	1454	50	1404
Day 5	1042	40.6	1283	41	1242
Day 20	670	41.2	801	21	780

T½ 15.0 days (fig. 1).
T½ 22.5 days. Column 5 extrapolated to zero time at 1448 cpm/ml.

diminishing zero blood isotope content for that day may be read directly from that graph and inserted in column 4 (table I). By subtracting that from the total isotope content of the sample in column 3, the counts from the recently labeled cells are determined, recorded in column 5 and plotted semilogarithmically.

Method II. Correct the *total* counts present in each sample of the repeat study to 50% hematocrit (table II, col. 3) *without* subtracting the zero isotope level. Plot the total counts on semilogarithmic graph paper. The resulting slope, although based on numerically inaccurate values for each individual population, reflects the mean life span of all tagged cells in the circulation as well as would attempts to differentiate the two components, as by definition both have the same survival when this method is employed. Should one wish to estimate each population, as when red cell volumes are determined, then after using method II the appropriate values should be diminished as in method I.

Erroneous Calculation Procedures: If the significance of labeled red cells in the baseline sample of the repeat survival study is not understood, errors in calculating the latter are probable.

Table II

Tabulation of isotope counts existing during the repeat survival study when red cells labeled for the repeat procedure and those present from the initial one have the same life span.
Calculation Method II

Repeat study samples	1 Total counts cpm/ml	2 HCT %	3 Total counts corrected to 50% HCT*
Zero Sample	43	41 7	52
Day 1	1160	42.5	1365
Day 5	765	40.6	942
Day 20	194	41.2	236

T½ 7.5 days. Column 3 extrapolated to zero is 1500 cpm/ml (initial study 52; repeat study 1448)

If the life spans of both cell populations differ and the initially labeled cells maintain their original longevity during the latter study ignoring the baseline isotope content by attributing the total counts to those cells labeled by the most recently administered quantity of isotope would be incorrect. It would erroneously lengthen the apparent repeat survival if the initial life span were long and falsely decrease it if the latter were short compared to the correct value of the repeat determination. Yet, tenfold increase in the baseline counts would alter the apparent life span only moderately If during the repeat study both cell populations truly have the same life span, but the initially labeled cells previously had a different one, then diminishing the zero sample counts according to that earlier disappearance erroneously lengthens the repeat survival if the initial one were short and falsely decreases it if the latter were long compared to the true value of the repeat study Only if the initial and followup values were truly the same would the use of these aforementioned procedures nevertheless result in the correct calculation of life span. However subtracting the zero counts in their entirety from each of the subsequent samples, as though the subject had never before received radioisotopes, would always diminish the apparent survival to greater extent than would those other erroneous procedures.

In general, the greater the difference between the true initial and repeat half-times or the greater the proportion of the total counts represented by the repeat study's zero sample, the greater the error if the proper calculation procedure is not used.

Discussion

Initially labeled erythrocytes which survive during a repeat study may continue to be destroyed at their original rate, or their life span may be altered to that of the cells labeled in the repeat procedure or to some other rate. Calculation methods to differentiate the cell populations have presently been developed for use in the first two instances. The third possibility would be impossible to recognize when a single isotope is repeatedly used to measure survival. However it is doubtful whether it would occur often. If it should, different isotopes must be used for each study

During the type of repeat study here considered, the radioactivity of the samples derives from two sources in varying proportions depending on the quantity of isotope administered on each occasion and the number of labeled cells of each population which survive in the latter sampling period. Therefore, the mean age of the cells is important. This is particularly true following exposure to certain hemolytic agents (17—19) including primaquine which operates preferentially against glucose-6-phosphate dehydrogenase deficient erythrocytes more than 60 days old (20)

If an initial survival study were performed after an acute exposure to a hemolytic factor acting against older red cells and a repeat study were done later when most susceptible cells had been destroyed, each cell population would survive differently from the other Likewise, if an initial life span determination is done under normal circumstances, followed by experimental exposure to an agent affecting immature cells the initially labeled cells, exclusively mature at that latter time, would not be affected. In both situations, extending the original sampling period could not truly reflect the ensuing altered rate of cell destruction. New survival studies would be needed for correct assessment of the hemolytic status. Substances such as folic acid antagonists (21) or diseases such as pernicious anemia (22) cause the formation of short lived erythrocytes having an intracorpuscular defect. These would circulate for a while following removal of the harmful factor or effective therapy of the disease. At that time, they would still hemolyze abnormally whereas many of the cells labeled during that subsequent procedure would be young ones having a normal life span. Calculation method I would be indicated in all the aforementioned situations.

Method II should be employed if following an initial determination of life span there is exposure to a variety of hemolytic agents (22—24) or diseases (25) or the development of blood loss. These factors would alter the survival of the originally tagged cells to that of those administered for the followup study

Repeated studies of leukocytes (26 27) platelets (28—30) fibrinogen (31) beta and gamma globulins (32 33) haptoglobin (34) and coagulation factor VIII (35) have been performed. The kinetics of some of the latter have been followed by chemical assay procedures not employing radioisotopes. Yet, correction for previously administered protein present at the time of a repeat determination is as applicable as when isotopes are used.

One of the earliest papers on erythrocyte life span using radiochromium (36) stated that a study may be repeated in 50—60 days when complete elution had occurred. However if different isotopes are employed for each determination (37) or an Ashby procedure is performed for one (14 38) so that each red cell population is readily identifiable, no such interval need exist.

The principle of estimating the initial isotope or dye persisting during subsequent determinations has been utilized in serial iron absorption, red cell, plasma and extracellular fluid volume experiments (39—41) It was first applied to erythrocyte survival by Hughes Jones (42) who performed serial disappearances of labeled red cells administered to the donor before and after periods of storage. During the latter radiochromium from the previous study was still present. He said, 'It was assumed that this 'background ^{51}Cr would disappear from the circulation at the same rate as ^{51}Cr-labelled normal cells It was necessary to make this assumption in order to make a correction for the "background counts on the day after injection However rather than assuming that the baseline isotope would disappear at the rate of normal cells it would have been more accurate to assume that the in vivo disappearance would have continued at its previously determined rate.

Summary

If a survival study is repeated while cells previously labeled with the same isotope circulate, the usual calculation method for determining life span must be modified because of the presence of ever-diminishing baseline radioactivity during the repeat procedure. Depending on whether the initially labeled cells maintain their original life span subsequent to the repeat administration of radioisotope or whether their survival has been converted to that of those labeled in the latter determination, as occurs in random destruction or blood loss, the use of either of two calculation methods is indicated. These methods have the advantage of being simple to perform as they involve semilogarithmic graphic plotting rather than complex mathematical analysis. However if the pattern of cell destruction cannot be anticipated then different labels must be used for each study

Résumé

Si la détermination du temps de survie est répétée tant que circulent encore des cellules radio-marquées précédemment au même isotope, la méthode pour calculer la durée de vie doit être modifiée, la radioactivité de base diminuant durant la répétition de la procédure. Suivant que les cellules radio-marquées initialement conservent leur durée de vie première après l'administration répétée du radio-isotope ou que leur survie devienne la même que celle des cellules radiomarquées lors de la détermination postérieure, comme cela arrive lors d'une destruction fortuite ou d'une perte de sang, l'une ou

l'autre des deux méthodes de calculation doit être employée. Ces méthodes ont l'avantage d'être simples, car elles ne demandent pas d'analyse mathématique compliquée, mais seulement un graphique semilogarithmique. Si cependant le mode de destruction ne peut être prévu, un marquage différent devra être employé pour chaque étude.

Zusammenfassung

Wenn die Bestimmung einer Überlebenszeit wiederholt wird, solange noch zuvor mit demselben Isotop markierte Zellen zirkulieren, muß die Methode für die Berechnung der Lebensdauer modifiziert werden, weil während der erneuten Bestimmung eine abnehmende basale Radioaktivität vorhanden ist. Je nachdem die zuerst markierten Zellen ihre ursprüngliche Lebensdauer bei Wiederholung der Isotopenzufuhr beibehalten oder aber ihre Überlebenszeit derjenigen der später markierten Zellen angleichen, wie dies bei gleichmäßigem Abbau oder bei Blutverlusten auftritt, ist eine der beiden Berechnungsmethoden anzuwenden. Diese Methoden besitzen den Vorteil der einfachen Anwendung, da sie anstelle komplizierter mathematischer Analysen nur eine semilogarithmische Aufzeichnung erfordern. Wenn aber die Art des Zellabbaues nicht vorausgesagt werden kann muß für jede Untersuchung eine verschiedene Markierung angewendet werden.

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Quantitation of the Erythropoietic Stimulus Produced by Hypoxia in the Plethoric Mouse

By Clifford W. Gurney, Pamela Munt, Ire Brazell and Diana Hofstra

The literature contains many references to the relationships between hypoxia and the production of the plethoric state (1–5) the effects of altitude on blood volume (6), reticulocyte response (7) and erythropoietin production (8). The present study is concerned with the minimum period of hypoxia required to activate the organ of erythropoietin production in the mouse.

Materials and Method

Carworth Farms No. 1 virgin female mice, 7 to 9 weeks of age, were given 1 mg of Proferrin intraperitoneally. Plethora was then induced by residence in a pressure chamber as originally described by Cotes and Bangham (9) except for approximately 0.25 hour at ambient conditions 2 times weekly for cage maintenance and change of water. Details of chamber operation and results obtained have been reported elsewhere (10). Following several days at ambient conditions, to permit maturation of erythroblasts already in cycle, a fresh wave of erythropoiesis was initiated by single injection of erythropoietin, a return to the chamber, or a combination of both. Forty-eight hours later 0.5 μc of $Fe^{59}Cl$ (specific activity approximately 35 mc/mg of iron) in volume of 0.2 ml was administered intravenously. In 2 experiments, the radioiron was brought to the desired volume by addition of mixture of 1 part postphlebotomy anemic mouse plasma to 4 parts of saline. In the remaining experiments, the radioiron was brought to volume with saline. Seventy-two hours later a measured volume of blood from the thoracic aorta was taken for scintillation well counting. From activity of standard prepared at the time of radioiron injection, and assuming a blood volume of 7% of the body weight for these animals, the percentage of the injected radioiron in newly formed peripheral red cells was determined (10, 12). It is assumed that this value is proportional to effective erythropoiesis, since both the radioiron incorpora-

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* Operated by the University of Chicago for the United States Atomic Energy Commission.

Erythropoietin was obtained from the Hematology Study Section of the United States Public Health Service. It was derived from anemic sheep plasma and represented 720-fold purification of raw plasma. Its biological activity is expressed in units as defined by Goldwasser and White (11).

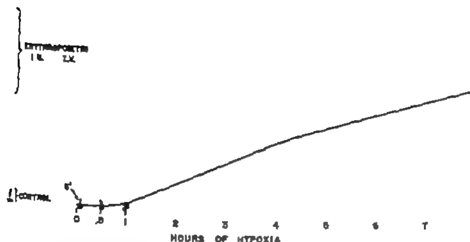


Fig. 1

tion of newly formed erythrocytes (12) and the reticulocyte response in plethoric mice (13) are functions of the amount of erythropoietin administered to these animals over wide range of dose.

Results

In the first experiment, mice were kept in hypoxia chambers for 28 days, at a pressure of 15 inches of mercury or approximately 0.5 atmosphere. After 4 days at ambient conditions, mice were re turned to chambers for such variable periods that all the animals could then again be removed from the chambers simultaneously. Figure 1 shows the percentage of Fe^{59} incorporation in individual animals after zero time, 5 minutes, 30 minutes, 1 hour 4 hours, or 8 hours in the chamber or after intravenous administration of 1 unit of erythropoietin. A clear erythropoietic effect can be noted after 4 hours of hypoxia, and a greater erythropoietic response at 8 hours.

RESPONSE TO HYPOXIA AND ERYTHROPOIETIN IN THE HYPOXIA-INDUCED PLETHORIC MOUSE

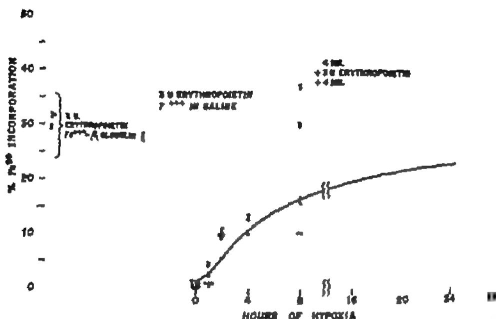


Fig. 2

In the second experiment, after 26 days of hypoxia mice were returned to the chamber for 0, 1 2, 4 8 or 24 hours. Again the return to different chambers was scheduled so that all animals would be finally removed at 4 days after their initial removal. One group of mice was then removed from the chamber given 3 units of erythropoietin subcutaneously and immediately returned to the chamber for an additional 4 hours. A second group of mice received radioiron in saline instead of in an anemic mouse plasma-saline solution. All the other mice in this experiment received radioiron in anemic mouse plasma-saline solution. Minimal stimulation of erythropoiesis was observed in 5 of 9 mice following exposure to 0.5 atmosphere for 1 hour and, as seen in figure 2, erythropoiesis increased with longer periods of hypoxia up to 24 hours. Erythropoiesis was more than twice as great after 2 consecutive periods of 4 hours of hypoxia separated by injection of 3 units of erythropoie-

RESPONSE TO HYPOXIA OR ERYTHROPOIETIN IN THE HYPOXIA-INDUCED PLETHORIC MOUSE AT VARIOUS TIMES AFTER CESSATION OF PROLONGED HYPOXIA

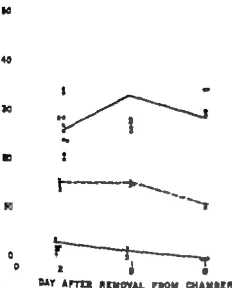


Fig. 3

tin, than after a continuous 8-hour period of hypoxia. There was no significant difference in radioiron uptake when radioiron was given in anemic mouse plasma-saline solution.

In the third experiment, after 22 days at 0.5 atmosphere and varying times at ambient conditions, some animals were returned to the chamber for 6 hours at 0.5 atmosphere, others were given 2 units of erythropoietin subcutaneously. Groups of untreated plethoric animals were used as controls. All radioiron was given in an anemic plasma-saline mixture. Figure 3 demonstrates the response to a standard hypoxic stimulus or to a standard dose of erythropoietin administered on the second, fifth, or eighth day at

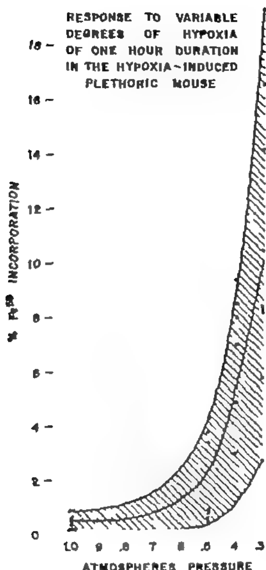


Fig 4

ambient conditions following removal from the chamber Between the second and eighth days, erythropoietic activity decreased slightly in the control animals, from an average value of 3.5 % iron incorporation to 0 4 %. There was no clear difference of response to a fixed 2 unit dose of erythropoietin, or to 6 hours at 0.5 atmosphere during this interval.

A series of experiments was designed to explore more intense hypoxia. After 22 days of exposure to 0.5 atmosphere, and 5 days of ambient conditions, groups of 13 plethoric mice were returned to chambers for 6 hours at different pressures. Ten non-acclimatized control mice of the same age accompanied each group of plethoric mice in order to ascertain the degree to which acclimatization enhanced survival. Mice previously acclimatized at 0.5 atmosphere can survive for 6 hours at 0.33 atmosphere but not at 0.25 atmosphere, while 4 of 10 non-acclimatized mice failed to survive 0.33 atmosphere. That the erythropoietic stimulus produced by 6 hours of hypoxia is related to the severity of the hypoxia is demonstrated by the radioiron incorporation, which averaged 9.5 % after exposure to 0.5 atmosphere and 20.9 % after exposure to 0.33 atmosphere. In this and the following experiments radioiron was administered in saline, instead of in anemic plasma-saline mixture.

We next examined the erythropoietic response following return to an environment of pronounced pressure reduction for a shorter interval of time. After 24 days at 0.5 atmosphere and 4 days at ambient conditions, 3 groups of 10 mice were returned to chambers for 1 hour at pressures indicated in figure 4. Eight control animals were not returned to the chamber. Some animals showed an erythropoietic response, and others failed to respond to 1 hour's exposure to 0.5 atmosphere, but all animals exposed for 1 hour to environments of 0.4 or 0.33 atmospheres showed a clear erythropoietic response. Although individual responses within each group varied widely, the average erythropoietic response increased rapidly as the pressure decreased from 0.5 to 0.33 atmospheres.

In the final experiment, after 33 days exposure to 0.5 atmosphere and 4 days at ambient conditions, groups of mice were returned to the chamber at either 0.25 atmospheres or 0.27 atmospheres pressure. Non-acclimatized mice of the same age were also subjected to the shorter periods of exposure, to assess survival enhancement by acclimatization. A previous prolonged exposure to hypoxic conditions was associated with a substantial degree of protection against the lethal effect of short periods of intense hypoxia. At 0.25 or 0.27 atmospheres, exposure for 20 minutes or less resulted in very slight stimulation in a few animals within the group, and since many of the animals failed to respond, standard deviations of the mean of these groups were high. At these pressures,

30 minutes was sufficient to produce a clear erythropoietic stimulus, and the response was greater at 45 minutes.

Discussion

Normal mice approach a new hematological equilibrium after approximately 3 weeks at a pressure of 0.5 atmosphere, a condition simulating an altitude of 18,000 feet (10). Longer periods of hypoxia do not further increase the hematocrit of blood from the tail vein, although the red cell mass and the plasma volume may continue to increase after the hemoglobin concentration has stabilized in a hypoxic environment (14, 15). It seems likely that once the plethoric state is established, an environmental pressure of 0.5 atmospheres no longer produces the striking hypoxic stress exerted by exposure without previous acclimatization. Following return to ambient conditions, the abundance of blood relative to need is such that erythropoiesis is reduced markedly.

We presume, but cannot yet prove, that the erythropoietic stimulus resulting from a brief return to 0.5 atmospheres is entirely dependent upon endogenous erythropoietin elaboration, although alternative mechanisms might be implicated. As yet it has not been shown that erythropoietin is always responsible for activation of erythropoiesis. STOHLMAN (16) does not agree that erythropoietin is the regulator of normal erythropoiesis, and an alternative mechanism that operates under normal circumstances might also account for erythropoiesis when animals adapted to 0.5 atmosphere pressure are returned to these 'normal' conditions. However such an alternative mechanism has not yet been demonstrated and the simplest postulation at this time is therefore that the erythropoietic activity observed in the animals returned to the hypoxia chamber is a consequence of the endogenous elaboration of erythropoietin.

In order to elicit demonstrable erythropoiesis in animals presumably elaborating no erythropoietin it appears that the hypoxic stimulus must be operating for approximately 1 hour when it is of the same intensity as that to which the animals were previously exposed. The stimulation produced by 1 hour 0.5 atmosphere, and by shorter intervals with more severe hypoxia indicate that even shorter periods are sufficient to initiate an erythropoietic stimulus, and presumably for the elaboration of erythropoietin. Some ani-

mals show a slight response to as little as 15 minutes exposure to severe hypoxia.

A sudden return to 0.5 atmosphere undoubtedly constitutes an erythropoietic stimulus, but it appears unlikely that it is comparable to that experienced by animals not previously conditioned. The stimulus experienced by previously adapted mice in 24 hours is somewhat less than that produced by a single *subcutaneous* injection of 3 units of erythropoietin. A dose response curve should make it possible to estimate the erythropoietic stimulus for any altitude and any period sufficiently short to approximate the time over which a single injection is active.

A number of ancillary questions have also been investigated during the present study. First, the necessity for injecting radioiron bound to mouse iron binding protein was considered. By previous bleeding of donor mice, plasma with an unsaturated iron binding capacity of 250 γ% was obtained, and a small amount of this plasma was sufficient to insure complete binding of radioiron of high specific activity. This precaution appears to have been unnecessary since we found the average radioiron incorporation following 3 units of erythropoietin was 29.7% of the half µc injected when given with mouse plasma, and 31.2% when given as $Fe^{59}Cl_3$ in saline. We therefore abandoned anemic plasma-saline mixture in favor of normal saline for diluting radioactive ferric chloride.

The degree to which the bone marrow might become refractory to erythropoietin, and the organ of erythropoietin elaboration might become refractory to a hypoxic stimulus after a prolonged period of decreased activity are of some importance in determining the optimal time for administration of the erythropoietic factor and may also be relevant to mechanisms of production of erythropoietin. We found little if any change either in elaboration of erythropoietic factor or in response to exogenous erythropoietin over the 6-day period. A simple demonstration of erythropoietin elaboration or marrow sensitivity in the first 48 hours following cessation of the prolonged hypoxic stimulus is impossible only because maturation of erythroblasts continues for several days following removal from the chamber and high baseline results are therefore obtained in unstimulated animals. Between the fourteenth and seventeenth day after removal from the chambers the hematocrit falls to less than 55% below which endogenous erythropoiesis may be resumed (un-

published data). Since the optimal assay of erythropoietin or the erythropoietic effect of a short period of hypoxia requires 5 days, it is not practical to extend the study beyond the eighth day after removal from the chambers unless the conditions of preparing the plethoric state are altered or fortified by blood transfusion.

Finally, if the erythropoietic response in plethoric animals returned to a hypoxic environment is the consequence of elaboration of erythropoietin, it is of interest to consider whether this response is limited by the amount of erythropoietin elaborated, or by the sensitivity of the bone marrow under hypoxia. While there is as yet no definitive answer to this question, the response in animals receiving 3 units of erythropoietin preceded and followed by 4 hours of hypoxia is strikingly greater than in animals exposed for 8 hours to hypoxia, and is slightly greater than the response observed in animals receiving 3 units of erythropoietin alone, suggesting that at least in a mildly hypoxic state, the bone marrow responds to erythropoietin in proportion to the amount of erythropoietin elaborated. Since the precise duration of activity of intravenously administered erythropoietin is not known, the summation of erythropoietin and hypoxia must be observed at longer intervals to exclude the possibility that the injected erythropoietic factor persists in the plasma or the bone marrow and exerts its primary stimulatory effect after removal from the chamber. This work will be reported at a later time.

Summary

Plethoric mice were subjected to short periods of hypoxia and subsequently employed for the bioassay of erythropoietin elaborated. Production of erythropoietin is demonstrated after one hour in hypoxic environment to which the animals have previously been exposed for 3 weeks, and is apparent in some mice after only 15 minutes exposure to severe hypoxia. Up to 24 hours exposure, the magnitude of the erythropoietic response increases with time, and for a constant interval of exposure the response increases with decreasing atmospheric pressure. In animals subjected to short periods of severe hypoxia survival is enhanced by previous adaptation to prolonged but less intense hypoxia.

Résumé

Chez des souris pléthoriques qui avaient été soumises à une hypoxie de courte durée l'érythropoïétine fut déterminée par dosages biologiques. La production d'érythropoïétine peut être démontrée après une heure d'exposition à des conditions hypoxiques,

après que l'animal y ait été soumis auparavant pendant 3 semaines. Elle est apparente chez quelques souris après 15 minutes seulement d'exposition à une forte hypoxie. Jusqu'à une durée d'exposition de 24 heures, la réponse érythropoïétique va en augmentant. Elle s'accroît également si le temps d'exposition reste le même, mais que la pression atmosphérique baisse. Chez des animaux soumis à de courtes périodes de forte hypoxie, le temps de survie est prolongé par une adaptation préalable à de longues périodes d'hypoxie moins forte.

Zusammenfassung

Bei plethorischen Mäusen wurde nach kurzdauernder Hypoxie das Erythropoietin biologisch bestimmt. Die Bildung von Erythropoietin ist nach einer Stunde unter Hypoxie-Bedingungen nachzuweisen, denen die Tiere zuvor während 3 Wochen ausgesetzt waren. Sie tritt bei einigen Mäusen bereits auf, nachdem sie nur während 15 Minuten einer schweren Hypoxie ausgesetzt worden waren. Bis zu einer Expositionsdauer von 24 Stunden nimmt die Reaktion der Erythropoese zu und steigt bei gleichem Intervall der Exposition mit abnehmendem atmosphärischem Druck an. Bei Tieren, die kurzen Perioden von schwerer Hypoxie unterworfen werden, lässt sich die Überlebenszeit verlängern durch vorhergehende Gewöhnung an länger dauernde aber weniger intensive Hypoxie.

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The Relation of Ultrastructure to D[illegible] thesis in Human Leucocytes

I. Atypical Lymphocytes in Phytohaemagglutinin Cu[illegible] Infectious Mononucleosis

By D R. Inman and E. H Co[illegible]

It is now well recognized that in res[illegible] a variety of stimuli and in a number of different speci[illegible] animals small lymphocytes are capable of modifying their st[illegible] e and dividing the most characteristic change in their morph[illegible] gy is an increase in size associated with a marked affinity for py[illegible] The activation of small lymphocytes in various homograft reactions (9 24) are examples of this type of response in vivo In vitro activation of human lymphocytes may be brought about by culturing them with phytohaemagglutinin (PHA) (18) and by culturing the cells of sensitized individuals with antigens such as diphtheria toxoid (8) or tuberculin (21)

In a preliminary investigation of the ultrastructural modification of human small lymphocytes which is induced by culturing them with PHA (7) we noticed that there is a close similarity between the ultrastructure of the transformed lymphocytes and the cells seen in infectious mononucleosis as described by other workers (19 25) A common feature of both these populations of cells is that a high proportion of them at any time are synthesizing DNA.

In the experiments described in this paper these studies of human lymphocytes stimulated by PHA have been extended. By the use of a combined electron microscopic and autoradiographic technique the structure of cells in DNA synthesis has been compared with that of cells not synthesizing DNA (G_1 and G_2) A similar

Wellcome Trust Senior Clinical Research Fellow

approach has been made in the study of infectious mononucleosis and the results of these two investigations have been compared.

Materials and Methods

Sources of cells

1. Normal peripheral blood leucocytes were cultured in Parker 199 tissue culture medium with PHA (Difco Laboratories Inc., Detroit, U.S.A.) using the method of Moorhead et al. (17). Other samples of normal lymphocytes were obtained from the thoracic ducts of patients during thoracotomy; some of these lymphocytes were cultured as above and examined at 20 and 72 hours after culture.

2. Peripheral blood from 4 patients suffering from infectious mononucleosis was obtained by venepuncture and taken into heparinised bottles.

Preparation of cells for autoradiography and electron microscopy

Samples of the cells, either from the cultures or freshly isolated, were incubated at 37 °C with 10 µC/ml tritiated thymidine, [illegible] C/mM (Radiochemical Centre, Amersham, England) for 30 minutes. At the end of this time the cells from the tissue cultures were centrifuged at [illegible]500 rpm for 2 minutes, the supernatant drained off and the cells fixed with cold buffered osmium tetroxide (2%) for 60–90 minutes at 4 °C. After incubating with ³HT the infectious mononucleosis cells were first separated from the blood by centrifugation in a Wintrobe tube and the buffy coat layer removed and fixed with osmium tetroxide as above. At the end of the fixation period the cells were washed in distilled water, dehydrated in ethanol and stored overnight at 4 °C in a mixture of methyl and butyl methacrylate (25:75) containing 1% uranyl nitrate, 2% benzoyl peroxide and 3% dibutyl phthalate. They were then centrifuged and embedded in fresh methacrylate solution. Polymerization was effected by heating to 60 °C for [illegible]–48 hours.

Thick-thin sectioning technique

All sections were cut on an A.F. Huxley ultramicrotome (Cambridge Instrument Company Limited) using either glass or diamond knives. Initially a series of thick sections (0.25 µ) was cut from each block and examined under phase contrast microscopy until a region in the block was reached which contained an adequate number of leucocytes. The block face was then trimmed to approximately 0.25 mm square. Thin sections suitable for electron microscopy were cut and mounted on a carbon-coated grid. A contiguous ca. 0.25 µ section was then cut, mounted on a glass slide and the methacrylate removed with chloroform in preparation for autoradiography at the light microscope level.

Autoradiography

The overall labelling of the cells in the various experiments was assessed by preparing standard Ilford K5 liquid emulsion autoradiographs of methanol-extracted cells taken just prior to adding the osmium tetroxide fixative.

The preparation of autoradiographs of methacrylate-embedded cells was based on the techniques described by Caro and Palade (3), by Hay and Revel (13) and by Granboulan (10). Briefly, this procedure consisted of coating the slides with Ilford L4 emulsion, diluted 1:8 with distilled water. The coated preparations were dried and



Fig. 1. Cells from 72-hour phytohaemagglutinin culture. Semi-thin section autoradiograph. b) Low-magnification electron micrograph mosaic of the contiguous, ultrathin section. Cells 1, 2 and 3 are synthesizing DNA. Cells 4, 5 and 6 are not synthesizing DNA. Cell 7 is in mitosis.



Fig 2. Infectious mononucleosis.) semi-thin section autoradiograph. b) Low magnification electron micrograph of the contiguous, ultrathin section. Cell 1 is synthesizing DNA. Cell 2 is not synthesizing DNA. At left is granulocyte.

stored at 4 °C 3 weeks exposure was required. After exposure the preparations were developed either in Kodak D19B or Microdol X developer and fixed in Amfix (May and Baker Limited, Dagenham, England) The emulsion was removed by immersing the slides in 0.05N NaOH for 1 hour The slides were then washed and lightly stained t room temperature w th 1% toluidine blue in 1 aqueous borax, differentiated in tap water cleared in xylol and mounted in DePeX (Edward Gurr Limited, London, England)

Correlation of light micrographs with electronmicrographs

The stained thick-section autoradiographs were examined by either phase contrast or bright field microscopy and low-power photographic map of the whole preparation made. All the labelled (DNA-synthesizing) cells were identified and marked on the map. The contiguous thin section was then examined in Siemens Elmiskop I at beam voltage of 60 kV with 50 μ objective aperture. The grids ere surveyed at magnification of 200 and each leucocyte photographed t higher magnification. The absolute identity of the DNA-synthesizing cells was later confirmed by the identical

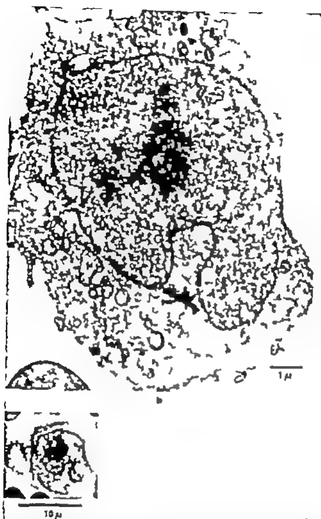


Fig. 3. DNA synthesizing cell from 72-hour phytohaemagglutinin culture.) Semi-thin section autoradiograph. b) Electron micrograph of the contiguous, ultrathin section.

morphology of the cell in the thick and thin sections and the pattern of the surrounding leucocytes and erythrocytes (fig. 1, 2 and 3

Cell size

A Leitz projection microscope was used to obtain an image of whole-cell preparations on tracing paper. The images of individual cells were then cut out and weighed to give an indication of cell size



Fig. 4. Size range of cells in phytohaemagglutinin cultures. Above: cells not synthesizing DNA (198 cells measured). Below: cells synthesizing DNA (89 cells measured). The hatched rectangle shows the size range of small lymphocytes in the same cultures.

Results

PHA-activated Cells

The ultrastructure of the cells in cultures of peripheral blood lymphocytes activated by PHA has been well documented (14, 16, 27). The dominant features are the appearance of a highly elaborate system of organelles and free ribosomes in the cytoplasm together with nuclear changes of varying degrees. The present experiments have been designed to show whether any of these modifications of the cellular organization are particularly related to active DNA replication by the cell. This question has been examined in cultures labelled with ^{3}HT after 72 hours growth. Three cell states are considered in the following analysis: small lymphocytes as identified by light microscopy, activated lymphocytes not incorporating ^{3}HT and activated lymphocytes incorporating ^{3}HT (fig. 3).

Cell size. No useful estimate of the size of a whole cell or whole organelles can be obtained from single electron micrographs due to the thinness of the sections. Figure 4 shows the distribution of the size measured by the projection method (see Methods) of whole cells in a 72 hour culture labelled with ^{3}HT.

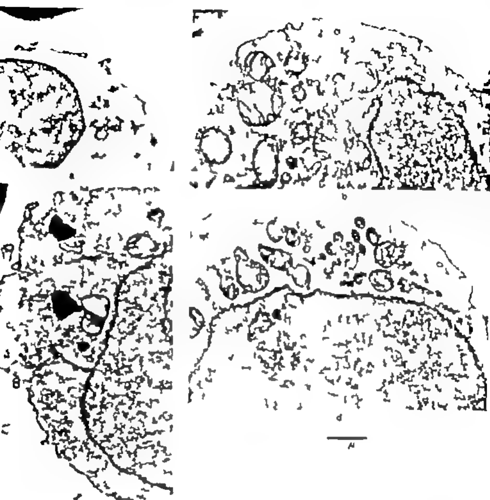


Fig. 5. Electron micrographs of normal and DNA synthesizing cells) Normal lymphocyte from 24-hour culture without phytohaemagglutinin b)) and d) cells synthesizing DNA from [illegible] hour culture containing phytohaemagglutinin.

Nuclei. Small lymphocytes showed no nuclear changes as compared with cells cultured without PHA or freshly isolated from the blood or thoracic duct lymph. The chromatin of these cells was dense and the two components of the nuclear double membrane were regularly spaced (fig. 5a). Small nuclear pores were present somewhat infrequently. Out of 19 activated negative cells examined

14 had similarly dense chromatin in the remainder the chromatin was more loosely packed 13 of these cells had regularly spaced nuclear double membranes but the nuclear pores appeared to be more frequent than in small lymphocytes. In 20 out of 22 ³HT positive cells the chromatin was very loosely packed though it was frequently more dense at the periphery than in the centre (fig 1 and 3) In all of these positive cells the nuclear double membrane showed a marked degree of irregularity as shown in fig 3 5 6 and 7 The separation of the two components was variable resulting in a beaded appaerance and was always greater than in the normal lymphocyte in many instances the outer membrane showed short tubular evaginations and it was frequently fragmented. In many of these cells one or more large nuclear pores were present (fig 6) occasionally nuclear material appeared to be entering the cytoplasm through these pores.

Nucleoli Small lymphocytes were not seen to contain nucleoli whereas they were present in about one quarter of the electron micrographs of the activated negative cells examined the nucleolar material in these cells ranged from dense to diffuse Nucleoli were seen in more than half of the electron micrographs of the activated positive cells, in some of these the nucleolar material presented a clumped or 'bobbined form (fig 3)

Mitochondria. Small lymphocytes contained an average of 3 mitochondria per electron micrograph section, the maximum seen in any one cell being 7 These mitochondria were of medium size and had regular though sometimes branched, cristae mitochondriales. Activated negative cells contained an average of 7 mitochondria (maximum 14) less than half of which appeared normal, the remainder being of bizarre form. Activated cells in DNA synthesis contained an average of 12 mitochondria (maximum 25) many of these were grossly swollen or elongated and contained cristae of bizarre form (fig 5 and 7)

Cytoplasmic RNA The density of the ribosomes in the cytoplasm was graded on the scale 0 to ++++ In the small lymphocytes the quantity present was never greater than + and was generally considerably less than this. In transformed negative cells the average density of the ribosomes was + in the transformed positive cells the average density was greater than ++ and sometimes as high as ++++ Practically all the ribosomes present

Table I

Haematological data of patients with infectious mononucleosis.

Patient	Age	Sex	Total W.B.C. /cmm	Lympho-cytes /cmm	Polymorphs /cmm	Percentage of W.B.C. labelled with ^{3}HT	Paul-Bunnell test
V. L.	20	F	10,000	5,440	3,600	6.0	1 3584
P. R.	22	F	11,000	3,850	4,290	[illegible]	1 1792
M. B.	21	F	7,000	5,200	1,600	9.2	1 896
A. L.	26	M	10,000	5,600	3,700	8.4	1 3584

Percentage of cells labelled after incubation with ^{3}HT for 30 minutes.

Key to Abbreviations used in figures.

G Golgi apparatus
L lipid containing body
M mitochondrion
MVB multivesicular body
N nucleus
NE nuclear envelope irregularities (see text)
N nucleolus
P abnormally large nuclear pore
p micropinocytotic vesicles
V filled vesicles, possibly containing glycogen

were in the free form either singly or arranged in rosettes of varying number.

Endoplasmic reticulum. Rough endoplasmic reticulum was completely absent in the cytoplasm of the small lymphocytes but was present in trace amounts in about half the activated cells whether synthesizing DNA or not. Smooth endoplasmic reticulum was present in trace amounts in half the small lymphocytes examined. It was difficult to estimate the quantity of smooth endoplasmic reticulum in the activated cells since their cytoplasm contained large numbers of microvesicles. It was our impression, however, that this system of cytoplasmic membranes was more elaborate in activated cells and particularly so in those synthesizing DNA.

Golgi zone. When this region was present in the plane of the section it was always found to be very poorly developed in small lymphocytes but showed a moderate degree of development in activated non-synthesizing cells. Activated cells synthesizing DNA often had elaborate Golgi zones; centrioles were occasionally present.

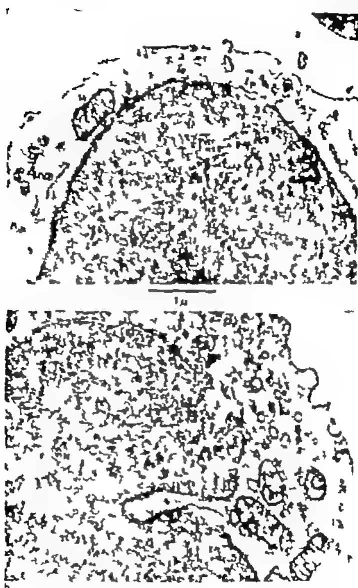


Fig 6. Electron micrographs of DNA synthesising cells.) and b show regions of the same cell in 72-hour phytohaemagglutinin culture

Other cytoplasmic properties

Some evidence of micropinocytosis was seen near to the cell membranes of small lymphocytes in culture whether PHA was present in the medium or not. In the activated cells however this

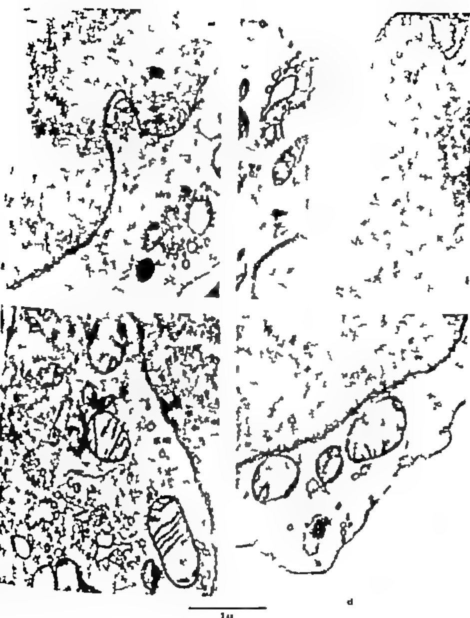


Fig 7 Electron micrographs of DNA synthesizing cells. The four parts of the figure are all of cells from 72-hour phytohaemagglutinin cultures.) and B are overlapping regions of the same cell A B There is staining artefact in and b)



Fig. 8. Electron micrographs of cells from 72-hour culture containing phytohaemagglutinin.) Cell 1 is normal activ ted lymphocyte. Cell 2 contains numerous bnormal cytoplasmic structures of unknown significance (see text). b) and) Higher magnification



Fig. 9 Electron micrograph of cell from 20-hour culture containing phytohaemagglutinin. The bnormal nuclear envelope and the high cytoplasmic ribosome content suggest that this cell has undergone considerable transformation No contiguous autoradiograph available.

micropinocytosis was greatly increased and was no longer confined to the region immediately inside the cell membrane. In the cells synthesizing DNA, tubules and lines of vesicles of micropinocytotic origin were sometimes seen extending from the periphery deep into the cytoplasm In those cells where marked micropinocytosis was a prominent feature of the cytoplasmic organization multivesicular bodies (24) were also significantly increased in number compared with normal small lymphocytes.

Vesicles containing an unidentified substance, possibly glycogen, were occasionally present in activated, non-synthesizing cells and were frequently seen in cells synthesizing DNA (fig 5d, 6a and d) These filled vesicles were not seen in small lymphocytes.

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of cell 1 The ucleus and nuclear membrane show the same bnormalities as DNA synthesizing cells The cytoplasmic structures are bounded by discontinuous membranes and contain mbrane-bounded osmiophilic bodies, myelin forms, vesicles, granular and filamentous components

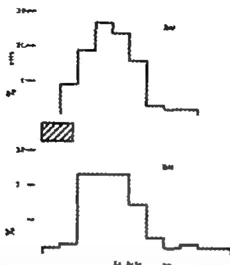


Fig. 10. Size range of lymphocytes in infectious mononucleosis. Above: cells not synthesizing DNA (101 cells measured). Below: cells synthesizing DNA (104 cells measured). The hatched rectangle shows the size range of small lymphocytes in the same preparation.

In some cultures cells were observed which contained numbers of cytoplasmic structures containing osmiophilic bodies and bounded by a discontinuous membrane (fig. 8). Some of these structures contained myelin forms, some contained empty vesicles as well as osmiophilic bodies, others contained fine granular or filamentous material. The nuclear chromatin and nuclear envelopes of these cells showed much the same form and degree of abnormality already described in DNA-synthesizing cells.

Short-term PHA cultures. The structural features associated with activated cells were not seen in cells from 12 hour cultures containing PHA but were present in occasional cells from 20-hour cultures. Fig. 9 shows a cell from a 20-hour culture in which the nuclear changes are apparent. No contiguous autoradiograph was available to show whether this cell was synthesizing DNA, but other autoradiographs have shown that small numbers of DNA-synthesizing cells are present after 20 hours in PHA culture.

Infectious Mononucleosis

The haematological data of the 4 patients investigated in this study are shown in table I. The distribution of the sizes of the

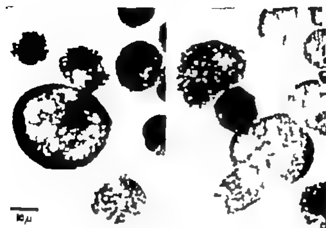


Fig. 11. DNA synthesizing cells in infectious mononucleosis. Composite autoradiograph of cell smear to show the size range of the positive cells (Patient P.R.).



Fig. 12. Electron micrograph of cells from infectious mononucleosis. Many features of transformed cells are present (see text) but no contiguous autoradiograph was available to show whether this had progressed to the stage of DNA synthesis.

various mononuclear cells in the peripheral blood in the patient P. R. is shown in fig. 10.

The ultrastructure of the small lymphocytes in the peripheral blood of these patients was normal. Many of the features seen in activated lymphocytes in PHA cultures and described above were present in the atypical lymphocytes isolated from the blood of patients with infectious mononucleosis (fig. 2 and 12). Certain differences existed, however, and these are outlined in the following paragraph.

From fig. 10 and 11 it can be seen that DNA synthesis cannot be related to cell size. A few of the cells synthesizing DNA were within the size range of normal small lymphocytes, the average diameter of the DNA-synthesizing cells was about twice as large and a few cells were observed to be about three times the size of small lymphocytes. This size distribution was equally true of atypical cells not synthesizing DNA. The chromatin changes in cells synthesizing DNA were similar to those present in DNA-synthesizing cells in PHA cultures, but the chromatin density and distribution in the nuclei of the atypical non-synthesizing cells were more akin to those of normal lymphocytes. The nuclear membrane changes in the DNA-synthesizing cells were as pronounced as those seen in synthesizing cells cultured with PHA. The density of free ribosomes in the cytoplasm of resting atypical cells and the higher ribosome density in those cells synthesizing DNA was very similar to that seen in the PHA-treated cells (fig. 2). The increased frequency and irregular morphology of the mitochondria was similar to that seen in the lymphocytes activated in vitro, as was the elaboration of the Golgi zone, though this zone did not reach a comparable degree of complexity. Micropinocytosis was a feature of the cytoplasm of the atypical lymphocytes but was only very marked in the DNA-synthesizing cells. No membrane bounded osmiophilic bodies were seen in the cytoplasm of the atypical lymphocytes in infectious mononucleosis. There was no detectable difference between the ultrastructure of cells incubated for 30 minutes to produce ^{3}HT labelling and cells transferred directly from patient to fixative.

Discussion

The method of combining autoradiography with electron microscopy involving the use of contiguous thick and thin sections

has the obvious disadvantage that the localization of the radioactivity is limited to the resolution of the light microscope. For resolution of a higher order electron autoradiographs must be prepared (4 10 13)

In the present problem, however the resolution needed was only that necessary to identify cells which had incorporated tritiated thymidine into their nuclei. Under these circumstances the thick thin method had certain advantages. Due to the thickness of the section and the consequent increase in the amount of radioactivity present a positive autoradiograph could be obtained after 2–3 week s exposure, whereas an equivalent ultrathin section autoradiograph required considerably longer A further advantage of this method is its use when the frequency of the radioactive cells in the total population in the preparation is low their position is first established on the thick-section autoradiograph and they can then be found with ease on the thin section in the electron microscope (30)

In the PHA activated cells all the changes of morphology the induction into and the progress through DNA synthesis occurred in vitro. In the atypical lymphocytes of infectious mononucleosis morphological changes were established and DNA synthesis was in progress in a proportion of the cells at the time they were sampled from the patient s blood. These cells were submitted to a 30-minute incubation with tritiated thymidine but this did not appear to have any effect on their morphology as their appearance was identical with that of cells fixed at once and was similar to those described by REINAUER (25) The ultrastructure of the atypical lymphocytes, arising on the one hand from a predominantly in vitro system and on the other predominantly in vivo, was much the same. It seems probable therefore that changes seen in the PHA activated cells were not a unique response to the combination of the PHA stimulus and the in vitro conditions per se. Cells with the same pattern of ultrastructure have been observed to arise in the lymphoid tissues in vivo when these tissues are reacting to certain antigenic stimuli (2, 22) It is only in PHA cultures, however that it has been firmly established that it is the growth and transformation of small lymphocytes that gives rise to these atypical cells (5 15 16)

The incorporation of tritiated thymidine into the DNA of the cells in PHA cultures and into the atypical lymphocytes in infectious mononucleosis has been shown to be exclusively associated with

DNA replication (6, 11) Furthermore, the cells synthesizing DNA in these two systems have the normal DNA content ranging from the diploid to the tetraploid value there is no evidence of polyploidy

The size of the cells synthesizing DNA in infectious mononucleosis varied over a considerable range but no difference in the ultrastructural organization of these cells was seen. At this stage, however we have no information as to whether these very small DNA-synthesizing cells constitute a distinct cell line in the general population. A survey of another 8 cases in the light microscope has shown that these small DNA-synthesizing cells are a common feature of the disease.

There was little evidence of micropinocytosis in normal small lymphocytes which had not been cultured. In cultured lymphocytes not subjected to the action of PHA some degree of micropinocytosis was taking place. This was markedly increased in cells acted upon by PHA and particularly so in cells synthesizing DNA. A similar situation existed in the atypical cells of infectious mononucleosis. Multivesicular bodies in small numbers are a normal finding in the cytoplasm of small lymphocytes (31) It seems possible that the increase in the number of multivesicular bodies seen in activated and DNA-synthesizing cells is a reflection of the increased degree of micropinocytosis in these cells since these organelles may be formed as a result of micropinocytotic activity

The nature of the cytoplasmic structures containing osmophilic bodies, myelin forms and vesicles seen in some cells in 72 hour *PHA* cultures is not known. The state of the *nuclei in these* cells suggests that they are not degenerate forms. It is possible that such structures arise from multivesicular body transformations in the manner suggested by ROBBINS AND GONATAS (26) for mitosing HeLa cells.

In the two cell systems under study three of the most significant changes which were seen to occur coincident with cells being in DNA synthesis were those in the density and distribution of chromatin those in the nuclear membrane and the intensification of the increase in cytoplasmic free ribosomes already evident in activated but non synthesizing cells.

Nuclear chromatin is a highly complex aggregate of various polymeric molecules—DNA, RNA, histones and other proteins. Their precise inter relationships during interphase is as yet unknown in the cells under examination, hence it is not possible to give a

simple interpretation of the chromatin density changes observed in cells synthesizing DNA. It may be that they are the result of reorientation of the DNA histone complex resulting from, or leading to replication. Alternatively they may reflect a redistribution of material associated with nuclear-cytoplasmic transfer of RNA (27) Ultrastructural cytochemical studies now in progress may help to give a more precise answer

The variations in the structure of the nuclear membrane associated with the different phases of growth and activation of the lymphocyte support the concept of BERNHARD (1) that this membrane is a highly complex organelle, actively involved in nucleo-cytoplasmic exchange mechanisms Indeed WATSON (29) has considered the nuclear envelope to be a specialized portion of the cytoplasmic membrane system. That vesiculation of the nuclear envelope occurs in cells not synthesizing DNA is now commonly recognized (1) However we have seen this appearance only very occasionally in normal cultured small lymphocytes and then not to any marked degree, whatever the angle of section. The fractionation of the outer nuclear membrane seen in DNA-synthesizing cells may possibly represent a very early stage in the breakdown of the nuclear envelope which is known to occur in mitosis. These early breakdown changes, if such they are, in the nuclear envelope of DNA-synthesizing cells resemble the appearance of the early prophasic and immediately post-divisional reforming nuclear envelope in cells of the sea urchin (12) Another possibility is that the nuclear membrane changes observed in activated DNA-synthesizing cells are wholly or partially connected with nuclear-cytoplasmic RNA transfer This explanation and the presence of exceptionally large-diameter pores in the membranes of these cells would support the suggestion of SIRLIN (27) that the presence of gaps larger than pores in the nuclear membrane or its disorganization (not breakdown) at particular periods of cell differentiation may facilitate exchange (of RNP or ribosomes)

Interphase can be considered to be divided into three main periods, G_1 S and G_2, DNA synthesis occurring only in the S period (15) Cell growth, on the other hand, tends to be continuous throughout interphase. The changes we have seen appear to be marked in cells which are in the S period of interphase. From our experiments, however it cannot be stated whether these changes are induced by the replication of DNA in the nucleus or are events

which, though occurring in interphase, are coincidental with this aspect of nuclear function.

Finally it should be emphasized that in both the cell systems studied the 'lymphocytes' were in a very atypical state. The nuclear membranes seen in the activated cells are clearly abnormal as compared with the small lymphocyte but this type of organization is not unknown in certain types of secretory and neoplastic cells.

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Summary

The ultrastructure of activated and DNA-synthesizing human lymphocytes in PHA cultures and in infectious mononucleosis was studied. A 'thick-thin' sectioning technique of combined autoradiography and electron microscopy was employed. Both cell types had very similar ultrastructural organization. Cells in DNA synthesis were observed to have) increased content of cytoplasmic 'free' ribosomes, b) Golgi zone elaboration and associated increase in numbers of mitochondria and vesicularity c) alteration of chromatin density and distribution, d) nuclear membrane changes.

Résumé

L'ultrastructure de lymphocytes humains activés et synthétisant de l'ADN a été étudiée dans des cultures de phytohémagglutinine et dans la mononucléose infectieuse. Une technique de débitage en coupes épaisses, puis minces, a été employée en combinaison avec l'autoradiographie et la microscopie électronique. Les deux types de cellules montrèrent une organisation très semblable de leur ultrastructure. Dans le stade de la synthèse de l'ADN, les constatations suivantes ont été faites: a) le contenu de ribosomes cytoplasmatiques «libres» est augmenté. b) une zone de Golgi est formée avec augmentation associée de mitochondres et de vésicules.) la densité et la répartition de la chromatine sont altérées. d) la membrane des noyaux est altérée.

Zusammenfassung

I Phytohämagglutinin-Kulturen und bei Mononucleosis infectiosa wurde die Ultrastruktur aktivierter und DNA-synthetisierender menschlicher Lymphozyten untersucht. Es wurde dazu eine «Dick-Dünnschicht»-Technik mit einer Kombination von Autoradiographie und Elektronenmikroskopie verwendet. Beide Zelltypen zeigten sehr ähnliche Organisation ihrer Ultrastruktur. Im Stadium der DNA-Synthese wurde daran folgendes festgestellt: a) ein erhöhter Gehalt an «freien» Ribosomen, b) Ausbildung einer Golgi-Zone und gleichzeitig Mitochondrien und Bläschenbildung) Veränderungen von Dichte des Chromatins, d) Veränderungen der Kernmembran.

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RETICULOCYTES PER 1000 ERYTHROCYTES

MATURATION FACTOR FOR ERYTHROCYTES MEASURED IN TERMS OF INCREASE OF RETICULOCYTES.

DAYS BEFORE INJ. INJECTIONS OF FACTOR ON ALTERNATE DAYS

1 2 3 4 5 6 7 8

1A

PER CMM BLOOD

MATURATION FACTOR FOR GR. MYELOCYTIC LEUKOCYTES

LEUKOCYTES PER CMM OF BLOOD

GRANULOCYTES PER CMM OF BLOOD

DAYS BEFORE INJ. INJECTIONS OF FACTOR ON ALTERNATE DAYS

1 2 3 4 5 6 7 8

1B

LEUKOCYTES

LYMPHOCYTES

1C

Fig 1A B and C. Examples of increase in hematopo- activity after injections of fraction C-etc. (A and B lymphocytosis effect of fraction C-4 (C)

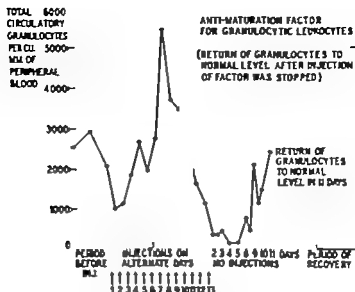


Fig 2. Example of response of peripheral leukocytes after injections of C-3 fraction. Granulocytes disappeared from the blood stream in 20 days. Recovery of maturation required 11 days after injections were stopped.

to fourteen. Prior to and after administration of fractions, counts of leukocytes, erythrocytes, thrombocytes, reticulocytes, values of hemoglobin, microhematocrit and differential counts of blood smears were done. All animals were killed and tissues were subjected to gross and microscopic studies. Cells in bone marrow were counted in 100 to 1000 fields depending on cellularity with 4 mm objective and 10 × ocular

C-1 was further fractionated by continuous flow electrophoresis and the subfraction containing albumin subfraction was injected into six rabbits.

Results

Fraction C-rec., composed of 4 protein with albumin comprising 50 when injected, produced an increase of hemoglobin by an average of 2 grams erythrocytes by a million, hematocrit by 7 and reticulocytes by 4 7 Granulocytes were increased by an average of 6 000 cells and the leukocyte content three times of normal. Mature granulocytes were 87–90 of leukocytes. Immature erythrocytes increased by 18 to 25 percent with an increase of the total. C-1 fraction, composed of 2.3 of total serum proteins, when injected showed effects similar to but to a lesser degree than with fraction C-rec.

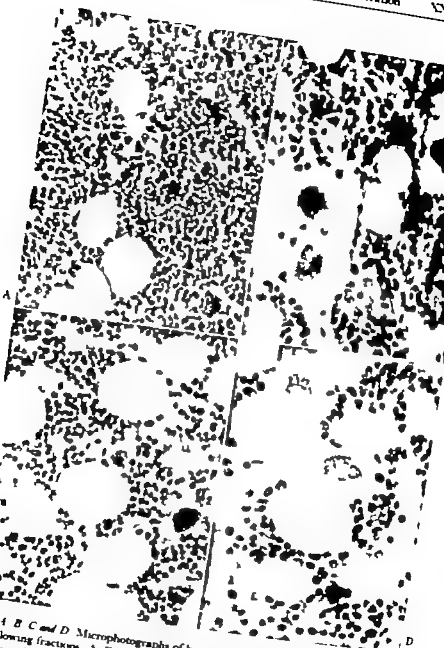


Fig 3 A B C and D Microphotographs of bone marrow of rabbits injected ith one of the following fractions A Fraction C-rec was associated with large number of poly morphonuclear leukocytes and erythrocytes. 190. B. Fraction C-3 produced suppression of production and maturation of granulocytes. × 225. C. Fraction C-4 did not alter the bone marrow 190 D Fraction C-5 induced lack of maturation and decrease of production of granulocytes and erythrocytes. × 225.

Fraction C-2 was inert. Fraction C-3 with 20 % proteins and albumin comprising 75 %, when injected reduced polymorphonuclear leukocytes fourfold without reduction of total leukocytes. Lymphocytes replaced granulocytes. Mature granulocytes were suppressed in bone marrow with a moderate increase of erythrocytes by 20 to 30 % (figs. 2 and 3B) Lymphoid tissue showed follicular hypoplasia.

Fraction C-4 with 31 % of protein and 38 % albumin, when injected, increased lymphocytes with total leukocytes varying from normal to threefold increase. The bone marrow had a normal cell content (figs. 1 C and 3 C) Lymphatic tissue was hyperplastic with immature lymphocytes. The splenic follicles were hyperplastic.

Fraction C-5 with 39 % of protein and 26 % albumin, when injected showed a decrease of hemoglobin by 50 % loss of two million or more erythrocytes, reduction of 10 to 11 % of hematocrit and a 95 % reduction of granulocytes with normal total leukocytes. Lymphocytes replaced granulocytes (fig 3D) Bone marrow was hypercellular with absence of mature granulocytes.

Subfraction of C-1 by continuous flow electrophoresis, with 16 % protein of which albumin was 81 % and 19 % made up of mostly α_2 when injected doubled or tripled the number of granulocytes. The bone marrow showed maturation of granulocytes and an increase of erythrocytes, mirroring the effect of total C-1 fraction.

Discussion

Humoral control of blood cells involved several mechanisms. There is one mechanism concerned only with expulsion of leukocytes from marrow to replace those in the blood stream. STEINBERG AND MARTIN (1 2, 7) determined that a substance labeled expulsion factor' probably released by disintegrating leukocytes, triggered the bone marrow to expel leukocytes to replenish the blood stream. Studies of YOFFEY (3) and CRADDOCK et al. (4) indicated the marrow to be the primary site for leukocyte replacement. In other studies, STEINBERG AND MARTIN (5 6) presented a second factor probably secreted by the spleen and other lymphatic tissue, labeled anti-expulsion factor which held expulsion to a physiological level The investigations of GORDON et al (7) PATT et al (8) and MENKIN (9) led to similar conclusions WEISBERGER et al. (10)

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Mechanisms of Leukocyte Production and Release

IV Factors Influencing Leukocyte Release from Isolated Perfused Femora of Rats with Chloroleukemia

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Burton S Dornfest
Albert S Gordon[2] and Francis C. Monette

This laboratory has been interested for some time in problems relating to leukocyte mobilization and release The nature of leukocyte discharge from normal isolated hind legs and femora has been extensively studied (2, 3 4 5 10) and we have recently investigated the pattern of leukocyte release from isolated perfused legs of rats with the Shay chloroleukemia (12) Comparisons with normal preparations revealed a diminished release of all types of white blood cells from the chloroleukemic hind legs. Since the entire leg was being perfused, the leukocyte contribution from the femoral bone marrow per se was not assessable. Isolated chloroleukemic femur preparations were perfused in the present series of experiments in an effort to determine the actual marrow leukocyte contribution.

Materials and Methods

Transplantation Procedure

The Shay chloroleukemia, an acute myelogenous leukemia has been maintained in our laboratory by an intraperitoneal injection of leukemic cells into 80—100 g rats of modified Long-Evans strain. Tumors were harvested t three-week intervals and the material loosely homogenized and filtered through glass wool. Sterile pyrogen-free saline was used as diluent in all instances. The final suspension contained 100×10^6 chloroleukemic cells per ml. Each rat received $20 \quad 10^6$ cells via intraperitoneal injection.

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Test Animals

Ether-anesthetized 220—200 g male rats were injected in the right jugular vein with 10 × 10⁶ chloroleukemic cells, prepared as described above. All leukemic rats used in these studies were inoculated ten days prior to perfusion. Normal control rats were of the same strain, sex and initial body weights.

Preparations of Femurs

Femurs were removed with care from rats anesthetized with 30 mg of sodium pentobarbital per kg of body weight. Five mg of heparin in 0.2 ml of physiological saline were injected into each test animal 5 minutes prior to amputation and the femurs were prepared as described by Dornfest et al. (3).

Perfusion Technique

A detailed description of the perfusion apparatus has been presented by Dornfest et al. (3). In essence, a rotor type peristaltic pump circulates the perfusate through the femur capped at its extremities with rubber tubing. The perfusate enters through the proximal nutrient foramen and the foramina in the proximal trochanteric fossa. The blood which passes through the femur is drained from the foramina located at the distal extremities and collected in calibrated test tube allowing flow rates to be determined. Perfusates were not recirculated in the experiments cited below.

Perfusate

Isologous blood was collected from male donor rats weighing between 300—400 g. Sodium heparin (0.2 ml of 2% solution) was added to each collecting syringe to prevent coagulation. Whole blood was centrifuged twice at 2000 r.p.m. for 20 minutes and the top leukocyte-containing layers removed. Leukocyte-depleted blood (50—150 WBC/mm³) was used in all experiments.

A leucocyte inducing factor (LIF) has been demonstrated in the plasma of leukocytapheresed (LAP) rats (7, 8, 9, 1). In one experiment leukocyte-depleted blood obtained from LAP donor rats was substituted for the normal leukocyte-depleted perfusate 30 minutes after start of the perfusion.

Parameters Studied

Perfusate. At frequent intervals the perfusate was sampled for hemocytometric counts, blood smears, and hematocrit determinations. Leukocytes were identified on smears stained with May-Grünwald and absolute numbers and percentages of these elements calculated.

Bone marrow. 1. *Quantitation.* A modification of the F ... and Gordon technique (6) was employed to quantitate femoral marrow of the perfused and the control, non-perfused contralateral legs. Each animal, therefore, served as its own control. Samples were taken for hemocytometer counts and smears. Nucleated erythroid elements were identified in smears treated with Ralph hemoglobin stain and lightly counterstained with hematoxylin. Myeloid and lymphoid elements were identified in preparations stained with May-Grünwald. Absolute numbers of the various cell types per milligram of femoral bone marrow were determined.

2. *Histology.* Representative femurs from normal and leukemic perfused and control preparations were fixed in buffered neutral formalin, decalcified in 10% EDTA solution, embedded in paraffin and sections were stained with hematoxylin and eo-

Table I

Mean numbers of leukocytes released from chloroleukemic and normal femurs perfused with leukocyte-depleted blood.

	Chloroleukemic femurs		Normal femurs
	Normal perfusate	LAP perfusate	Normal perfusate
No. of experiments	7	1	3
Perfusion time (min)	126	120	117
	(120—139)		(98—128)
Total perfusate (ml)	8.3	10 9	3.6
	(1.5—16 0)		(2.7—5.2)
Flow rate (ml/hr)	3.94	5.45	1.88
	(0.8—7.8)		(1.29—2.40)
Total perfusate cellular release ($\times 10^{-6}$)			
Mono[1]	11.56	6.01	5.77
	(6.27—26.70)		(4 96—6 68)
PMN[1]	6.95	3 71	8 63
	(3.45—9.00)		(5.32—10.90)
EOS[1]	0.18	0.18	0.23
	(0 05—0.40)		(0 10—0.39)
Chloro[1]	23.66	15.03	—
	(0.0—118.44)		
Total	42.38	24.95	14.64
	(11.33—150 05)		(10 60—16 95)
Perfusate cellular release (10^{-6}/ml)			
Mono[1]	1.62	0.55	1 77
	(0.73—2 67)		(0.95—1.90)
PMN[1]	1 46	0.34	2.73
	(0.22—4 53)		(1.00—3.60)
Eos[1]	0 02	0 02	0 69
	(0.01—0.04)		(0.04—1.30)
Chloro[1]	2.23	1.38	—
	(0.00—11.34)		
Total	5.33	2 29	4.58
	(1 47—15.00)		(2 04—6.06)

Denotes range.
Mono Mononuclear cells.
PMN Polymorphonuclear leucocytes.
Eos Eosinophils.
Chloro Chloroleukemic cells.

Results

Mean cellular perfusate release values for chloroleukemic and normal femur preparations are given in table I. From these data it is evident that total cellular release from chloroleukemic femurs was enhanced, due primarily to discharge of chloroleukemic elements. The large range of release values, however militated against a meaningful statistical evaluation In order to initiate perfusions in



Fig 1 Histological section of bone marrow from normal rat femur perfused with leukocyte-depleted blood for hours. Note the presence of distended marrow sinusoids (S) with no extravasation of blood × 300.

Table II

Mean femoral marrow cellularity (millions per mg) of perfused and contralateral non-perfused chloroleukemic and normal femur preparations

	Chloroleukemic	Chloroleukemic +LIF	Normal
	Perfused		
Lymph	0.540 (0.024—1 170)	0.168	0 991 (0.784—1.215)
PMN	0.382 (0.051—0 759)	0 190	0.472 (0.404—0.576)
Eos[a]	0.057 (0 004—0 089)	0.002	0.075 (0.050—0.100)
Chloro[a]	0 065 (0 030— 1.640)	1 719	—
Total nucleated cells	2.21 (1.60—2.77)	2.16	2.34 (1.98—2 80)
	Contralateral		
Lymph[a]	0 639 (0.013—1.816)	0.121	0 810 (0 710—0.850)
PMN	0.323 (0.149—0.406)	0.226	0.520 (0.470—0.570)
Eos[a]	0 060 (0 0—0.10)	0 020	0.098 (0 07 —0.12)
Chloro[a]	0 072 (0.009—1.39)	1.509	
Total nucleated cells	2.25 (1 86—3.10)	1.98	2.42 (2 08—2 94)

Denotes range.
Lymph Lymphocytes
PMN Polymorphonuclear leukocytes
Eos Eosinophils
Chloro Chloroleukemic cells

(The promyelocyte, myelocyte, metamyelocyte and nucleated erythroid cell content of the 10-day chloroleukemic rat bone marrow was comparable to that seen in marrows of normal rats and is therefore not included in this table.)

chloroleukemic femurs abnormally high pressures (80—160 mm Hg) had to be employed (normal range 30—50 mm Hg). Once perfusions were underway the mean flow rate through chloroleukemic femurs (3.94 ml/h) was greater than noted through perfused preparations (1 88 ml/h).

Although large numbers of cells were released from both normal and chloroleukemic femurs, the marrow compartments

Fig 2. Histological section of normal non-perfused, femoral bone marrow. Note the normal appearance of the marrow parenchyma and vasculature (× 300).



Fig 3. Histological section of bone marrow from chloroleukem with leukocyte-depleted blood for 2 hours. Note the appearance traversing mechanical pathways MP) through the chloroleukemic

were by no means depleted (table II) The slight decrease in cellularity per mg of shaft marrow from perfused femurs may well be due to the migration of cells from epiphyseal regions during the perfusion period. The addition of perfusate containing the LIF did not alter the pattern of flow rate and cellular release which remained similar to that seen in chloroleukemic femurs perfused with WBC-depleted blood from non LAP rats.

Histological examination of perfused normal femurs (fig 1) revealed an intact parenchyma with no evidence of extravasation of blood. In general the cell types and vasculature were similar to contralateral control femurs (fig 2) Perfused chloroleukemic femurs, on the other hand, showed marked extravasation of blood. It was noted, in these preparations, that the blood perfusing under the abnormally high pressures had created artificial pathways resulting in continued blood flow through the femoral marrow parenchyma. Destruction of the marrow integrity was associated with the presence of clumps of perfused red cells within the parenchyma (fig 3) The chloroleukemic marrow was homogeneous containing predominantly chloroleukemic elements and was largely devoid of marrow sinusoids and small blood vessels. The major arterial supply appeared intact (fig 4)

Discussion

The data presented indicate that greater numbers of leukocytes were released from chloroleukemic femurs perfused with leukocyte depleted blood than from normal preparations perfused in a comparable manner This pattern is different from that noted when isolated chloroleukemic whole hind legs were similarly perfused (12) A study of the experimental conditions, however has shown that abnormally high perfusion pressures were necessary to initiate blood flow through the chloroleukemic femurs. This was not the case with any of the normal femur perfusions. The usual criterion for determining marrow parenchyma disruption is the discharge of large numbers of immature elements into the perfusate In the present instance this criterion is no longer valid since liberation of immature (chloroleukemic) cells is considered physiological in the

Fig 4 Histological section of non-perfused femoral bone marrow from chloroleukemic rat. Note the compact marrow parenchyma composed largely of chloroma cells and the absence of normal vasculature. 300.

leukemic state. Concurrent histological observations explain the necessity of using high perfusion pressures when isolated femurs were employed. Due to the partial obliteration of the marrow sinusoids and small vessels, the perfusate in all likelihood could not traverse the marrow These observations strongly suggest that, in the intact leukemic animal, the marrow microcirculation is impaired, thus reducing the supply of oxygen and nutrients. PETRAKIS et al (11) have demonstrated that individuals suffering from acute leukemias exhibit an increase in intramedullary pressure. This elevation of pressure was thought to be due to an obstruction of venous return and an increase in the amount of blood within the marrow cavity In the present series of experiments, elevated perfusion pressures were necessary to overcome the increased resistance in the pathological marrows. These non physiological pressures succeeded in forcing a pathway for the perfusate, through the leukemic marrow parenchyma. Once a route had been established the perfusate traversed the femur with little difficulty The flow rate through leukemic femurs was, therefore as expected, greater than through normals It is known that precapillary sphincters regulate blood flow through capillary networks (14 15) The normal blood flow through femoral marrow is controlled by precapillary sphincters acting on marrow sinusoids (1) The apparent paucity of such elements associated with an increased flow rate suggest a mechanical defect in the perfused femur rather than a reflection of the normal physiology of the leukemic circulation The inability of the LIF to effect an increase in either flow rate or numbers of leukocytes released from the chloroleukemic femurs may be considered further evidence for an abnormal marrow circulation. It has been shown that the LIF induces a leukocytosis via augmentation of marrow blood flow probably by opening previously closed sinusoids (9) The absence of such structures from the marrow would thus be expected to obscure the effects of the LIF

The chloroleukemic state in the rat is characterized by major alterations in the femoral bone marrow vasculature. This is reflected by decreased leukocyte release from isolated chloroleukemic hind legs (12) and the necessity for employment of high pressures to perfuse isolated chloroleukemic femurs. It is suggested that the diminished leukocyte release from the leukemic bone marrow is masked in the intact animal by the release of normal leukocytes and chloroleukemic cells from extramedullary sites of production.

In the terminal stages of the Shay chloroleukemia the bone marrow becomes hypoplastic (13). The elevated circulating leukocyte levels which characterize the late stages of the pathogenesis are due, therefore, to mechanisms other than increased cellular production and release from the bone marrow. Unpublished observations reveal the spleen to be a site of extramedullary myelopoiesis. Investigations are currently in progress to determine the nature of the splenic contribution in different stages of the pathogenesis of the Shay chloroleukemia.

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Summary

Leukocyte release from isolated perfused femurs of rats with the Shay chloroleukemia was determined and compared with normal preparations. Histological studies show that alterations in femoral marrow vasculature developing during the course of the leukemia prevented physiological perfusions. Increased cellular release and augmented flow rates were due to artificial pathways created by abnormally high perfusion pressures. High peripheral circulating levels of leukocytes in the late stages of the Shay chloroleukemia were attributed to extramedullary sites of cell production and release.

Résumé

Chez des rats ayant une chloroleucémie de Shay, la libération de leucocytes par perfusion isolée du fémur a été déterminée et les résultats comparés à ceux obtenus avec des préparations de rats normaux. Des études histologiques ont mis en évidence des altérations des vaisseaux de la moelle du fémur qui se développent durant la leucémie et qui empêchent une perfusion physiologique. La libération accrue de cellules et l'augmentation de la perfusion sont provoquées par l'apparition de voies artificielles dues aux hautes pressions employées pour la perfusion. Le nombre élevé des leucocytes circulant dans les stades avancés de la chloroleucémie de Shay est dû à l'hématopoïèse extramédullaire.

Zusammenfassung

Die Abgabe von Leukozyten aus dem isoliert durchströmten Femur von Ratten mit Shay-Chloroleukämie wurde bestimmt und mit normalen Präparaten verglichen. Die histologische Untersuchung zeigte, daß Veränderungen der Vaskularisierung des Femurmarkes, die im Laufe der Leukämie auftraten, eine physiologische Perfusion verhinderten. Die Steigerung von Zellabgabe und Durchströmung war bedingt durch artefizielle Strombahnen, die zufolge der hohen Perfusionsdrucke entstanden. Hohe Werte zirkulierender Leukozyten in den Spätstadien der Shay-Chloroleukämie sind auf extramedulläre Blutbildung zurückzuführen.

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Studies in the Development of a Screening Test of Iron Storage Disease

By P. Biedermann, J. D. Fitzgerald and H. Keberle

In the normal person the urine contains some 0.05 to 0.1 mg of iron per 24 hours. The kidneys cannot normally excrete excess quantities of body iron. The introduction of iron chelating agents to clinical medicine (3) provided a method of enhancing the renal excretion of iron. The earlier chelating agents such as BAL or EDTA (5–8) caused only a slight rise in iron excretion in normal persons though they did enhance the renal excretion of iron in a small number of cases with iron storage disease.

The study reported here concerns the application of a new highly specific iron chelating agent, Desferrioxamine (DFO), to the problem of detecting excess iron stores in the human. The work is based on the hypothesis that there may be a difference in the renal excretion of iron after a standard dose of DFO between normal persons and those with excess iron stores.

It was necessary first to establish the pattern of response of a group of normal persons to a standard dose of DFO. It was considered that for clinical application, the test should be as simple as possible. In order to determine the optimal response to DFO the early part of the work was concerned with the measurement of the pattern of urinary iron excretion following a standard dose of DFO.

Materials and Methods

Initial studies were made in 20 healthy normal males and 11 females aged between 20 and 45 years of age. They all had normal haemoglobin and serum iron levels, as well as normal total iron binding capacity. The urine was normal in [illegible] subjects. The urinary iron was estimated on an over-night specimen. Desferrioxamine methane sulphonate (DFOM) 500 mg in 5 ml of water was given by deep intramuscular injection. The urine was collected accurately every 3 hours for 12 hours. There was no restriction on fluid or food intake.

Table I

The 3-hourly renal excretion of iron following 0.5 g of Deferrioxamine in 20 males.

No.	0–3 hours Urine ml	Iron mg	DFOM mg	3–6 hours Urine ml	Iron mg	DFOM mg	6–9 hours Urine ml	Iron mg	DFOM mg	9–12 hours Urine ml	Iron mg	DFOM mg
1	98	0.100	65.5	84	0.182	34.0	985	0.360	3.7	585	0.093	3.3
2	49	0.079	8.5	53	0.117	57.3	92	0.168	3.6	154	0.154	6.1
3	55	0.105	6.5	93	0.197	24.6	166	0.173	6.3	300	0.077	1.3
4	196	0.086	87.5	260	0.240	45.2	180	0.162	13.2	243	0.112	4.6
5	260	0.187	150.0	146	0.283	21.3	116	0.155	6.25	168	0.133	2.9
6	160	0.131	121.0	166	0.255	37.4	172	0.148	8.72	236	0.156	2.8
7	445	0.107	17.6	350	0.238	34.5	410	0.181	7.3	470	0.114	8.9
8	70	0.144	102.0	152	0.234	27.4	340	0.109	1.4	243	0.207	0.85
9	218	0.087	92.0	130	0.213	38.3	85	0.180	19.5	170	0.133	2.4
10	128	0.154	93.0	196	0.177	37.2	85	0.095	8.4	73	0.103	3.1
11	244	0.161	169.0	495	0.197	37.2	160	0.080	8.8	114	0.073	2.1
12	150	0.073	132.0	87	0.000	34.3	90	0.024	6.7	244	0.045	7
13	156	0.203	102.0	240	0.120	42.7	220	0.088	10.7	264	0.073	3.8
14	268	0.034	97.0	127	0.102	13.3	265	0.033	6.2	353	0.177	1.7
15	92	0.092	93.0	106	0.165	49.7	138	0.055	10.1	205	0.193	3.3
16	157	0.031	61.0	205	0.175	73.3	215	0.150	26.5	133	0.083	8.1
17	141	0.107	107.0	380	0.175	32.7	60	0.034	1.2	200	0.080	1.6
18	143	0.137	53.0	147	0.156	32.7	452	0.542	30.2	185	0.093	3.0
19	93	0.043	127.0	140	0.190	55.8	257	0.308	20.8	465	0.139	1.4
20	100	0.120	60.9	205	0.189	22.0	135	0.146	14.6	244	0.150	5.6
	161	0.110	86.3	185	0.180	39.5	230	0.175	10.7	253	0.126	3.1

DFOM = Deferrioxamine

All urine was collected in iron-free containers. The serum iron and total iron binding capacity were determined by RAMSAY method (6); the urinary iron by wet ashing method (KEBERLE, unpublished). All estimations were done in duplicate and the serum values were determined within 6 hours of collection.

Results

The 3 hourly renal excretion of iron in males, foll DFOM intramuscularly is given in table I. There was iron excretion over the whole 12 hours with the occurring in the 3 to 6 hour period. The average cretion over the expected excretion per 3 hours is It can be seen that DFOM caused a 3 to 5 times in urinary iron. It seemed therefore that for the purposes a test, the best post injection collection period would However not only is this rather impractical point of view but it can be seen in table I that

Table II

Comparison of the average 3 hourly iron excretion in 20 male subjects before and after 500 mg of Desferrioxamine.

Time in hours	Expected excretion (mg %)	Post-Desferrioxamine excretion (mg %)	Factor
0– 3	0.03	0.11	3.6
3– 6	0.033	0.18	5.4
6– 9	0.04	0.175	4.3
9–12	0.045	0.128	2.7

Table III

6-hour renal excretion of iron in 20 males and 11 females after 0.5 g of Desferrioxamine.

	No.	ml of urine	mg of iron		No.	ml of urine	mg of iron
♂	1	182	0.282	♀	21	654	0.222
	2	102	0.196		22	870	0.540
	3	148	0.302		23	970	0.290
	4	456	0.326		24	815	0.294
	5	408	0.470		25	975	0.290
	6	326	0.386		26	700	0.322
	7	795	0.345		27	735	0.191
	8	222	0.378		28	330	0.165
	9	348	0.300		29	375	0.247
	10	264	0.331		30	400	0.040
	11	759	0.358		31	950	0.372
	12	257	0.075		Average = 0.26 ± 0.03 mg		
	13	416	0.325				
	14	393	0.136				
	15	198	0.237				
	16	362	0.306				
	17	521	0.282				
	18	290	0.390				
	19	233	0.233				
	20	305	0.309				
	Average = 0.29 ± 0.024 mg						

(11 12) achieved a higher iron excretion in the first 3 hour period. It was therefore decided that the optimal post injection collection period was 0–6 hours. For technical reasons only the 0–6 hour urine was collected in the female control group and the results of this together with 6 hour urine results in men are given in table III.

As a result of this experience it was then decided to investigate a family two of whose members had died of haemochromatosis.

The genealogical tree of this family is given in fig. 1. It can be seen that the study included two brothers and sister of the deceased haemochromatotic patients as well as the first and second generation siblings of the propositus. One of the family (D) had

Sibling S. Early form of haemochromatosis (most probably idiopathic). Haemosiderin granules present in the peribiliary localization, more pronounced in the periportal areas. Near the V. hepaticae there is an abundance of iron-free haemofuscin-like pigment. Both pigments exclusively present in the hepatocytes. The latter appear to be intact, although differences in nuclear size can be seen. There is no indication of fibrosis.

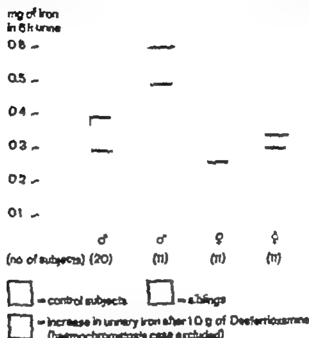


Fig. – Mean 6 hour urinary excretion of iron in normal subjects and immediate relatives of haemochromatosis patient following 0.5 g and 1.0 g of Desferrioxamine intramuscularly

Table 17
Comparison of results of DTPA test (10) and Desferal test in normal subjects

	DTPA	DFOM	DFOM (12)	DFOM (11)	DFOM (7)
Males	0.11 ± 0.08 (9 subjects)	0.29 ± 0.02 (20 subjects)	0.398 ± 0.13 (27 subjects)	0.28 mg (28 subjects)	0.7 mg (10 subjects)
Females	0.08 ± 0.07 (8 subjects)	0.26 ± 0.03 (11 subjects)	0.264 ± 0.1 (15 subjects)		

Sibling Q. This is a more advanced case with similar morphological features. There is mostly haemosiderin pigment, partially in larger lumps in both hepatocytes and Kupffer cells. Signs of focal cell degeneration are present. There is slight fibrosis of the portal tracts. There is very little iron-free pigment in the areas near the central veins.

The response of these two siblings to DFO was as follows
Sibling S 0.4 mg Fe after 0.5 g DFO 0.56 mg Fe after 1.0 g DFO

Table VII

Urinary iron excretion after 0.5 [illegible] of Desferrioxamine [illegible] various diseases.

Indication	Investigators: [illegible] (8) (48 h urine)	[illegible] (11) (6 h urine)	[illegible] (12) (6 h urine)	[illegible] (7) (6 h urine)	[illegible] (4) (6 h urine)
Hepatitis	3.4 mg (3 cases)	0.72 to 2.8 mg (28 cases)	0.87 mg (1 case)	0 6 mg (2 cases)	0.34 mg (1 case)
Various infections			0.5 mg (15 cases)		
Cirrhosis		0.06 to 1.8 mg (26 cases)		1.5 mg (5 cases)	
Haemochromatosis	10.0 mg (2 cases)	7.3 mg	4.0 mg (13 cases)	11.0 mg (2 cases)	
Porphyria cutanea tarda		0.3 to 0.8 mg (12 cases)	1.5 mg (3 cases) 0.4 mg (2 cases)		

Sibling Q 0 46 mg Fe after 0.5 g DFO 0.5 mg Fe after 1 0 g DFO The figures for the 0.5 g DFO response are just above our range for normal males. It may be noted that despite histological evidence of increased iron deposits the serum iron and total iron binding capacity were normal though the response to DFO was abnormal.

Discussion

In the 12 years since FOREMAN (5) first showed that iron chelating agents could influence the urinary excretion of iron, the majority of studies have been concerned with ascertaining the *effectiveness* of agents that enhance iron excretion. The study reported here was undertaken initially to obtain information on the pattern of response rather than the effectiveness in normal persons of a highly specific chelating agent, DFO After the intramuscular administration of 0.5 g of DFO there is always an increase in the total amount of iron excreted in the urine in the following 12 hours. The increase can be detected in the first 3 hours and reaches a maximum some 3 to 6 hours after the injection. There is, however no closely reproducible pattern of response. The variation between individuals depends partly on the urine flow though in several instances a lower urine volume in the second 3 hour collection period was associated with a higher iron excretion (Nos. 1 5 6, 7 9 13 and 17) In two individuals (Nos. 1 and 18) the highest iron excretion occurred in the third 3 hour urine collection period. In both instances this was associated with a very considerable

increase in urine volume, though in No. 18 there was also a rise in the urinary iron concentration.

In view of the known individual variation in DFO metabolism, the possibility that the variation in iron excretion was partly due to differences in DFO metabolism and excretion was also considered. The 6 hour urinary excretion of DFO varied from 7.3 % (No. 3) to 42 % (No. 19) of the administered dose, with a mean 6 hour excretion of 29.4 %. No correlation could be found between the excretion of DFO per 6 hours and the urinary iron excretion. Measurements were also made of the rate of enzymic inactivation of DFO in the plasma of the two most widely differing subjects Nos. 3 and 19 but no differences in this activity could be detected.

Other studies in normal persons Several other workers have studied the response to DFO in normal persons. Table VI compares their findings. It can be seen that the normal values of both WÖHLER (12) and WEWALKA (11) are similar to our own. This points to a degree of reliability in the test. STROHMEYER (7) noted a slightly higher response (0.74 mg) in 10 normal persons.

The members of the haemochromatotic family responded to DFO with 6 hour iron excretion values that fall within the upper normal range. It seems probable that the test in its present form is not sufficiently sensitive to detect with certainty early iron overload. However the DFO test results in the two clinically normal siblings with abnormal deposits of iron in the liver sections suggest that there is a degree of correlation even in early cases of iron storage disease. A point to be emphasized is that these siblings did not give a normal response. Had such a finding occurred it would have indicated a serious limitation to the test.

There can be little doubt that it would select undiagnosed cases of frank iron overload. It is suggested that, using the described method of urinary iron estimation, any patient who excretes more than 1.5 mg of iron in the 6 hour urine after 0.5 g of DFO intramuscularly is likely to have excess iron stores an excretion of more than 2.5 mg may be diagnostic.

Studies in pathological conditions Some interesting results have been obtained from the application of the test to some pathological conditions (table VII) Twenty-eight cases of acute hepatitis were studied by WEWALKA (11) These gave post DFO values between 0.75 mg and 2.8 mg of urinary iron per 6 hours. The values decreased to normal as the clinical condition improved, but subacute

and chronic cases of hepatitis had a persistentl[illegible] ated response above 0.5 mg Several cases of obstructive jau[illegible] gave normal values when the test was performed in the fir[illegible]ek, but gave values of 0.5 to 2.2 mg if the jaundice had beer[illegible] t for 3 weeks or more However NIEBS (4) has reported a[illegible]etion of only 0.34 mg of urinary iron per 6 hours in the stan[illegible] test in a single case of chronic hepatitis. Further in a case of c[illegible]ic cholangitis, he observed an excretion of 0.24 mg per 6 hou[illegible] He has not reported on studies in normal persons. STROHMEYER (7) observed a response of 0 6 mg in two cases of hepatitis, but this was within normal limits for his study UNSELD (9) studied the 48 hour urinary iron excretion following 500 mg of DFO in three cases of hepatitis, all of whom had elevated serum iron values. He found 48 hour urinary iron excretions of between 2 6 mg and 5 3 mg

WÖHLER (12) has made some interesting observations on the response to DFO in a variety of infections, including tuberculosis, polyarthritis, meningoencephalitis, poliomyelitis, and glandular fever He noted an enhanced urinary iron response to DFO (average 0.5 mg per 6 hours) together with a fall instead of the usual rise in 2 hour post DFO *serum* iron value There is no clear explanation of these interesting observations

The response in cases of cirrhosis of the liver has been very varied. WEWALKA (11) obtained figures ranging from 0 06 mg to 1.8 mg per 6 hours, but found increased haemosiderin deposits histologically in all cases excreting more than 1 2 mg per 6 hours. STROHMEYER (7) observed twice normal values in 3 out of 5 cases of liver cirrhosis. The response to DFO in cirrhosis will be influenced by the frequency and severity of haemorrhagic episodes.

As is to be expected the highest responses were found in cases of haemochromatosis. WÖHLER (12) found an average excretion of more than 4 mg of iron per 6 hours in 13 cases with only 1 case excreting less than 2.5 mg In 5 cases, WEWALKA (11) noted a response of more than 3 mg and in two cases STROHMEYER (7) observed excretion values of 15 mg and 7 6 mg per 6 hours respectively UNSELD (9) measuring the 48 hour urinary iron excretion, observed values over 2 mg in 4 cases of haemosiderosis, 16 7 mg in one case of haemochromatosis, and 4 0 mg in one case of secondary haemochromatosis.

DAGG et al. (1) have studied the response of normal, sideropenic and iron deficient patients to 600 mg of DFO They collected

the urine for 24 hours and their mean values for the normal patients were 0.75 mg, for sideropenic patients 0.4 mg and for iron deficient patients 0.12 mg. These preliminary studies indicate that a DFO test may have an application in detecting depleted iron stores as well as excessive iron stores.

A similar type of study is reported by WALSH et al. (10). These workers used intravenous diethylene triamine penta acetic acid (DTPA). They based their study on the observation of FAHEY et al. (2) that parenteral DTPA increased the urinary excretion of iron in cases of iron overload but not in normal subjects. WALSH administered 1.0 g of DTPA intravenously for 30 minutes and collected a single 5 hour specimen of urine. Table VI shows the 5 hour urinary iron excretion in normal subjects, after 1.0 g of DTPA intravenously and also the 6 hour urinary iron excretion after 0.5 g DFO. The response to DFO is about 5 times greater if it is accepted that the extra hour of urine collection is offset by the more efficient route of administration of DTPA. Their study also included the use of this test in 13 close relatives of haemochromatotic patients. All the relatives had raised serum iron levels and saturated transferrin values but only 6 were untreated. They responded with an excretion of 0.5 to 4.5 mg of iron in 5 hours. The difference in their material was too great to draw comparisons with the family which we have studied.

In conclusion, it is reasonable to state that there is a rough correlation between the total body iron stores and the response to a single injection of DFO. It seems very likely that the test described here would detect cases of iron storage disease amongst an unselected group of patients. Variation in laboratory technique for the estimation of urinary iron may make comparative studies difficult and more experience is necessary in the application of this test in a variety of pathological conditions.

Acknowledgements. We wish to express our thanks to Dr. LAWRY, Mr. FAIRN and M. B[illegible] for their technical assistance.

Summary

The urinary iron excretion was studied in 20 males and 11 females following a single injection of Desferrioxamin (DFO). It was found that the 6 hour urinary iron excretion following intramuscular injection of 0.5 g of DFO was 0.25 to 0.5 mg. The response of [illegible] siblings of a family 3 of whom had haemochromatosis was studied. The response of the family as a whole was in the high normal range. The application of the Desferal test is discussed and the results of studies in various pathological conditions is reviewed.

It is suggested that the Desferal test may be of value in the diagnosis of iron storage disease and possibly iron depletion states.

Résumé

L'excrétion urinaire de fer a été étudiée chez 20 hommes et 11 femmes après une injection unique de desferrioxamine (DFO). Durant les 6 heures suivant l'injection de 0,5 g de DFO l'excrétion urinaire de fer se monte à 0,25–0,3 mg. Chez 23 frères et sœurs d'une famille dont trois membres souffraient d'hémochromatose, les valeurs trouvées étaient à la limite supérieure de la normale. L'emploi du test au Desféral est discuté et les résultats d'études concernant différentes maladies sont passés en revue. Il est suggéré que le test au Desféral pourrait être de valeur dans le diagnostic des thésaurismoses du fer et probablement aussi dans celui des manques de fer.

Zusammenfassung

Bei 20 Männern und 11 Frauen wurde nach einer einmaligen Injektion von Desferrioxamin (DFO) die Eisenausscheidung im Urin untersucht. Innert 6 Stunden nach intramuskulärer Injektion von 0,5 g DFO betrug die Urineisenausscheidung 0,25–0,5 mg. Bei 23 Geschwistern einer Familie, von denen 3 eine Hämochromatose hatten, lagen die Werte an der oberen Grenze der Norm. Die Anwendung des «Desferal»-Testes wird diskutiert, und die in der Literatur mitgeteilten Befunde bei verschiedenen Krankheiten werden besprochen. Es wird vermutet, daß der Desferal-Test für die Diagnose von Eisenspeicherkrankheiten und wahrscheinlich auch von Eisenmangelzuständen von Nutzen sein kann.

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Erythrozyten werden 2800000 nur bei den Diskriminatoreinstellungen von 10 und 15 Einheiten erfaßt, entgehen also der Routinezählung, die bei 20 Einheiten durchgeführt wird.

Versuche die unterschwelligen Erythrozyten durch Höherstellen des Verstärkers über die Diskriminatorlinie von 20 Einheiten zu heben, führten zu keinem befriedigenden Ergebnis. Die erhaltenen Werte waren dann stets zu hoch, wahrscheinlich da auch die Impulse der Thrombozyten die Diskriminatorlinie überschreiten und mitgezählt werden.

Diskussion

Dieser Fall zeigt einmal mehr, daß auch die perfekteste Automatik nicht frei von Fehlerquellen ist, und daß man sich hüten muß, ein »automatisches« Ergebnis von vorne herein als absolut sicher anzusehen. Wenn auch in unseren Gegenden die Thalassämie eine Seltenheit darstellt, so wird man ihr bei der immer zunehmenden Einwanderung von Arbeitskräften aus den Mittelmeerländern doch wohl häufiger begegnen. Außerdem stellen stark mikrozytäre Anämien – besonders bei Säuglingen – keine Seltenheit dar. Auch in diesen Fällen ist ein ähnliches Versagen der elektronischen Zählung möglich.

Einen ausgesprochenen systematischen Fehler stellt diese Fehlerquelle jedoch nicht dar, da das Schirmbild den geübten Beobachter sofort auf eine Mikrozytose aufmerksam macht. Um die Frage zu klären, ob zu kleine Erythrozyten der Zählung entgehen, wird man als erstes eine zweite Zählung bei 15 Diskriminatoreinheiten durchführen, bei der die unterschwelligen Erythrozyten ein signifikant höheres Resultat ergeben. Beträgt der Unterschied zwischen den beiden Werten nicht mehr als 2%, so kann man mit Sicherheit annehmen, daß das Gerät alle Erythrozyten erfaßt hat. Ist der Unterschied höher, so liegen unterschwellige Mikrozyten vor. Man wird in diesem Falle am einfachsten eine visuelle Zählung vornehmen. Sollte eine neue Blutentnahme nicht möglich sein, so kann auch die erste Erythrozytenverdünnung (1:200) wie üblich in der Kammer gezählt werden.

Zusammenfassung

Am Beispiel eines Falles von Thalassaemia minor wird der störende Einfluß der Mikrozytose auf die automatische Erythrozytenzählung im Celloscope (Ljungberg) beschrieben. Mit Hilfe der Price Jones-Kurve der Erythrozytenvolumina wird gezeigt, daß die für diese Krankheit typischen, unterschwelligen Mikrozyten unter Routinebedingungen der Zählung entgehen, und somit zu niedrige Erythrozytenwerte vorgetäuscht werden.

Summary

The disturbing influence of microcytosis on automatic red cell counting with the Ljungberg celloscope is illustrated by means of case of thalassemia minor. The Price-Jones curv of the red cell volume reveals how the subliminal microcytes typical of this disease escape detection and lead to falsely low red cell counts.

Résumé

Les perturbations causées par une microcytose dans le compte automatique des érythrocytes à l'aide du celloscope «Ljungberg» sont démontrées en prenant comme exemple le cas d'une thalassémie mineure. La courbe des olumes érythrocytaires de Price Jones démontre que les microcytes subliminaux typiques échappent au compte t que, par conséquent, un nombre trop bas d'érythrocytes est simulé.

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Slight Increase of Fetal Hemoglobin in Apparently Healthy Negroes

By Bennett F Horton Dorothy A. Hahn and Titus H J Huisman

Several Caucasian families in which there are apparently normal members with a persistence of a slight increase of HbF have been reported by Betke and Marti (1 2) This abnormality was considered to be under genetic control. This paper reports the results of studies of two Negro families in which a slight increase of alkali resistant hemoglobin was observed in several apparently healthy members. Evidence will be presented that this fraction is identical to fetal hemoglobin, which seemed to be present in specific red blood cells.

Methods

The percentages of HbF in hemolysates were determined by the technique described by Betke et al. (3) values up to 1.2% were considered normal. The values to be reported are averages of 5 separate determinations with standard error of ± 0 1%. The distribution of HbF in the red blood cell population ('F-cells') was determined by the method of Betke and Kleihauer (4) Starch gel electrophoresis was carried out by the method of Smithies as modified for hemoglobin electrophoresis (5)

Two large hemoglobin samples, one obtained from case III, 5 with normal level of HbF and one from case III, 4, with an increased quantity of alkali resistant hemoglobin (fig 1) were treated similarly to Betke et al. alkali denaturation technique The residual hemoglobins were concentrated on CMC columns (6) and studied by determining the UV spectra using Beckman DK 2 Spectrophotometer by starch gel electrophoresis and by Amberlite IRC-50 chromatography using developer No. 5 according to Schroeder et al. (7 8, 9) Total hemolysates from the above mentioned two family members were also chromatographed on Amberlite IRC-50.

Routine hematological procedures were carried out by commonly used methods. The percentage of HbA_2 was determined by chromatography on DEAE-cellulose as described by Huisman and Dozy (10)

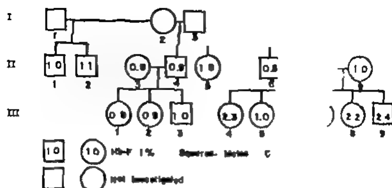


Fig 1 Pedigree to Family R. The figures presented refer the p ent alkali resistant hemoglobin.

Results

The pedigree of family R is presented in fig 1 The propositus (II 8) was a 38 year old Negro male referred to the Eugene Tal madge Memorial Hospital for possible sickle cell disease. His total Hb at the time of admission was 14.5 gm / with a hematocrit of 45 /. Starch gel electrophoresis revealed the presence of normal HbA with a small, but definite, quantity of HbF Eighteen members of this family were available for further study increased quantities (1.5 to 2.4 /) of HbF were observed in the propositus, in his sister and in 5 of his 6 children. When compared with the 11 family members with normal HbF no significant differences in the hematological values were observed (table 1) There was no evidence of an increased but compensated hemolysis as judged by the reticulocyte counts (ranging from 0 1 to 1 2 /) and normal bilirubin values. Also no significant differences in the values of HbA_2 (mean of 2.7 / for 7 patients with elevated HbF and mean of 2 6 / for 7 patients with normal HbF) were observed. Fifty / hemolysis was observed at NaCl concentrations ranging from 0.36 to 0 41 gm / for 6 cases with increased HbF and at 0 37 gm / for 2 normal individuals. Serum iron and iron binding capacity values of 95 to 170 μgm / and 363 to 591 μgm / resp. and a normal red cell morphology excluded iron deficiency

Blood samples from each member of this family were also examined for F-cells The results indicated that the fetal hemo-

and by an unequal distribution of HbF when increased. The observation presented in this paper suggest that the members of the Fam. R. and probably also of the Fam. L. are not heterozygotes of any of these thalassemia subtypes. The observation of a slightly increased level of fetal hemoglobin a deviation from normal, which seems to have a genetic basis, points to a persistence of the production of small but increased quantities of γ-chains during adult life without interference in the production of normal HbA containing red blood cells. The possibility that this genetic anomaly causes increased production of Hb Bart's (γ_4) in an affected newborn should be considered, although the evidence presented is insufficient to support this.

Acknowledgement. This study was supported by American Cancer Society grant no. IN75A and U.S.P.H.S. grant no. HE-05168

Summary

Two Negro families are described in which a slight increase of fetal hemoglobin (2 to 3) is found. The fetal hemoglobin in one of these families was studied by several techniques. The slight increase of fetal hemoglobin seems to occur in healthy individuals without hematological abnormalities. In the second family there were minimal hematological abnormalities associated with the occurrence of this slight increase of fetal hemoglobin. The propositus, a newborn, had an elevation of Hb Bart's.

Résumé

Rapport sur 2 familles de nègres dans lesquelles a été trouvée une légère augmentation de l'hémoglobine foetale (2–3). Dans une des familles, l'hémoglobine foetale été étudiée par différentes techniques. L'augmentation légère de HbF semble se trouver chez des sujets sains sans aucune anomalie hématologique. Dans la deuxième famille quelques anomalies hématologiques minimes étaient accompagnées d'une légère augmentation de l'hémoglobine foetale. Le propositus, un nouveau-né, présentait une augmentation de l'hémoglobine Bart.

Zusammenfassung

Es wird über 2 Negerfamilien berichtet, die eine leichte Vermehrung von HbF (2 bis 3%) aufweisen. Das fetale Hämoglobin der einen Familie wurde mit verschiedenen Methoden untersucht. Die leichte Vermehrung von HbF scheint bei gesunden Individuen ohne hämatologische Anomalien vorzukommen. Bei der zweiten Familie ging die geringe Vermehrung von HbF einher mit minimalen hämatologischen Veränderungen. Der Propositus, ein Neugeborenes, wies eine Vermehrung von Hb Bart auf.

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The Cells of Uterine Adenocarcinoma

By James W. Reagan and Alan B. P. Ng, Cleveland, Ohio

156 p., 79 fig., 6 tab., 1965. Fr./DM 32.–

In this era of Cellular Pathology knowledge of the cellular changes in disease has taken on a new importance. While much is known about the cells of squamous cell cancer in the uterine cervix and its antecedent lesions, knowledge of the cells originating in adenocarcinoma is more limited. This is a detailed analytical study of the cells which are derived from uterine adenocarcinoma. The cellular characteristics of the various types of adenocarcinoma are considered along with their significance. The effects of differentiation, extent, inflammation and necrosis are evaluated. The distinctive features of endometrial and endocervical adenocarcinoma are described as well as the cellular changes relating to adenocarcinoma, metastatic to the uterus.

Contents

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From the Medical Clinic of the University of Perugia
(Director: Prof. Paolo Larizza)

Studies on Some Factors Regulating the Energy Metabolism of Leukocytes*

By F. Grignani, A. Colonna, M. Martelli, Marisa Mastrodicasa and M. Tonato

The energy metabolism of normal and leukaemic leukocytes was recently dealt with in detailed reviews (32, 16, 17, 20). Leukocyte metabolism is mainly characterized by active aerobic glycolysis and a high glycolysis-respiration ratio. However, all the details concerning the mechanisms regulating these two metabolic processes as well as the interactions existing between them are not known. Moreover, the quantitative ratios between aerobic and anaerobic glycolysis as well as between glycolysis and respiration have been until now variously estimated (2, 3, 7, 8, 15, 16, 17, 20, 24, 27, 28, 31). All enzymes of the glycolysis and of the Krebs cycle were found in the leukocytes (2, 27, 28 to 30). However, we are lacking sufficient knowledge particularly as regards the enzymes of the Krebs cycle. Moreover, very little is known about the energetic efficiency of glycolysis and of respiration. Some investigations (23, 18) on the behavior of the two metabolic processes during phagocytosis and some other indications (15, 13) seem to attach greater importance to glycolysis.

Abbreviations: ADP: adenosine-5'-diphosphate; ATP: adenosine-5'-triphosphate; DNP: dinitrophenol; DOAP: Dihydroxyacetonephosphate; FDP: fructose diphosphate; F-6-P: fructose-6-phosphate; GAP: glyceraldehyde-3-phosphate; GP: glycerophosphate; G-6-P: glucose-6-phosphate; IAA: iodoacetic acid; IP: inorganic phosphate; PG: 3-phosphoglycerate; TRIS: Tris(hydroxymethyl)aminomethane.

A. L.: acute leukaemia; C. M. L.: chronic myelogenous leukaemia; C. L. L.: Chronic lymphatic leukaemia.

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A comparison between the metabolic behavior of normal and leukaemic leukocytes has not allowed to identify significant qualitative discrepancies. It is only known that the production of lactate and the uptake of O_2 are greater in normal than in leukaemic leukocytes.

The present investigations concern the mechanisms regulating glycolysis and respiration of normal and leukaemic leukocytes. The metabolic role of some substrates (glucose, succinate and glycerophosphate) and of inorganic phosphate was studied. The interactions between glycolysis and respiration under various experimental conditions and the activities of all glycolytic enzymes and of some enzymes involved in respiration were also investigated.

Materials and Methods

The leukocytes were isolated and purified from normal subjects or leukaemic patients by the technique described elsewhere (20). A cell population made mostly of granulocytes (90%) was obtained.

Cell fractions were obtained by the following method. The isolated leukocytes were suspended *in a very small amount of saccharose 0.25 M and homogenized in Potter* for 10 min at 3° C. The homogenate was then diluted with saccharose 0.25 M and centrifuged at *1500 x g in a refrigerated centrifuge.* Precipitate 1 (*PR 1*) and supernatant solution 1 (SN 1) were obtained. By centrifuging supernatant fluid 1 in refrigerated centri*fuge* 5000 g, precipitate 2 (*PR 2*) and supernatant solution 2 (SN 2) were got. Further attempts aimed at isolating endocellular particles (mitochondria, microsomes etc.) failed. All these particles are to be found in precipitate 2. Determinations on SN 1, SN 2 and PR 2 were accomplished.

Lactic acid production was determined in the leukocyte suspension. The cells were incubated in Warburg flasks at 37° C for one hour. The reaction mixture contained leukocytes suspended in Ringer without phosphates, 2.5 – 3 × 10^7, 10 mM glucose, phosphate buffer at a varying concentration, pH 7.4, 50 mM triethanolamine buffer pH 7.4, to 3 ml (variations of the reaction mixture will be indicated in the text).

Aerobic glycolysis was examined in an air atmosphere. A nitrogen atmosphere was created in the Warburg vessels for the analysis of anaerobic glycolysis.

The production of dihydroxyacetonephosphate and glyceraldehyde-3-phosphate was followed for one hour in Warburg respirometer utilizing leukocyte suspension or homogenate of packed leukocytes diluted to the initial volume with Ringer without phosphates. Determinations of lactate and triosephosphate esters were performed at *0 minute and at the 60 minute. As a difference it was calculated the production of metabolites*, which was expressed in μMol × min^{-1} and referred to 10^{10} cells.

The oxygen taken up by the leukocyte suspension or the cell fractions was measured at 37° C for one hour in Warburg respirometer in the presence of *Tris* buffer pH 7.4 and in an air atmosphere. *The substrates and phosphate added will be indicated from* time to time in the exposition of results. For the evaluation of oxygen consumption in the cell fractions the following incubation mixture was utilized: in the main chamber *Tris* buffer pH 7.4 8 mM, KH_2PO_4 10 mM, Na succinate 20 mM, Na fluoride 7 mM, cytochrome C 0.01 mM, ATP 2 mM, $MgCl_2$ 4 mM, preparation under consideration; in the side arm glucose 25 mM, crystalline Hexokinase Boehringer 0.01 ml; in the internal chamber NaOH 5 M 0.2 ml. The results achieved with leukocyte suspension were

expressed in μMol of O_2 taken up × $min^{-1}/10^{10}$ cells. In the case of cell fractions reference to 1 mg of protein, instead to the cell number was made.

Assay of Glycolytic Metabolites

1 ml of the incubation mixture was deproteinized with 1 ml of $HClO_4$ 6%. After 10 min at room temperature, the mixture was centrifuged and the supernatant fluid, neutralized with KOH 2 N was utilized for the determinations.

The lactate was assayed following HOHORST' method (22), the mixture of triosephosphate esters (glyceraldehyde 3-phosphate and dihydroxyacetonephosphate) according to the technique described by GRIGNANI et al. (20).

Enzyme Assays

) *Spectrophotometric:* The activity of glycolytic enzymes and some other enzymes of pentose shunt and the Krebs cycle was assayed in the leukocyte cytolysate prepared according to GRIGNANI et al. (20) or in the separated cell fractions (SN1 SN2 and PR2)

Hexokinase was determined according to GRIGNANI AND LÖHR (21) the fructose-6-P kinase according to BÜCHER (9) the aldolase according to BEISENHERZ et al. (6) the triosoisomerase according to BEISENHERZ (5) the glycerophosphate dehydrogenase according to BARANOWSKI (1), the glyceraldehyde-3-P dehydrogenase according to BEISENHERZ et al. (6) the 3-phosphoglycerate kinase according to BÜCHER (9) the enolase according to BÜCHER (10), the pyruvate kinase according to BÜCHER AND PFLEIDERER (11) the lactate dehydrogenase according to BEISENHERZ et al. (6) the glucose-6-P dehydrogenase according to KORNBERG AND HORECKER (25) the malate dehydrogenase according to BÜCHER AND SCHMASSEK (12).

The determinations were carried out by means of spectrophotometer Beckman DU A unit of enzyme activity is defined as the turnover of 1 μMol of substrate per minute; specific activity is defined as units per 10^{10} cells and/or 1 mg of protein.

b) Manometric. The activity of the succynic dehydrogenase according to KEBOWITZ AND LÜTTGENS (26) and of the glycerophosphate oxydase according to GRIGNANI et al. (19) was assayed in Warburg respirometer in the presence of toluidine blue.

The results were expressed in μMol of O_2 consumed per minute by 10^{10} cells. The protein content was determined measuring the absorption of samples at 280 and 260 nm following WARBURG AND CHRISTIAN' method (31).

Results

Table I shows the lactate production from normal and leukaemic leukocytes, under aerobic and anaerobic conditions, in the presence of varying concentrations of inorganic phosphate in the incubation medium. In all experimental conditions normal leukocytes produced more lactate than the leukaemic ones.

In normal cells inorganic phosphate increased markedly the glycolysis rate. The highest production of lactate was obtained with phosphate concentration of 30 μMol/ml. In contrast, in leukaemic leukocytes a higher rate of glycolysis was obtained in the presence of 3 and 11 μMol of inorganic phosphate per ml. More elevated

Table I

Lactate production (μMol/min/10^{10} cells) of normal and leukaemic leukocytes under aerobic and anaerobic conditions in the presence of varying concentrations of inorganic phosphate.

Inorganic phosphate mM		1		5		11		30	
Gas phase		Air	Nitrogen	Air	Nitrogen	Air	Nitrogen	Air	Nitrogen
Normal	M			8.86	23.03	16.00	25.00	24.45	31.05
leukocytes	σM			1.55	2.55	1.90	2.22	2.38	1.97
C. M. L.	M	9.75	16.80	13.20	19.05	12.90	19.90	8.00	13.02
	σM	1.42	1.17	2.50	[illegible]	2.55	0.55		
C. L. L.	M	3.30	11.20	5.10	11.70	4.40	15.00		
	σM	0.77	2.70	0.81	1.60	0.57	1.46		
A. L.	M	9.90	14.20	10.25	16.80	10.40	19.25		
	σM	2.30	1.49	0.70	2.60	2.90	2.00		

phosphate concentrations inhibited lactate production in chronic myeloid leukaemia.

In normal granulocytes the lack of inorganic phosphate affected mainly the lactate production under aerobic conditions the Pasteur effect, therefore, became significant as there occurred a deficiency of phosphorus ions. In leukaemic cells, and namely in those of chronic myeloid leukaemia, instead, a significant Pasteur effect was already found even at optimal concentrations of phosphate and was not markedly affected by the shortage of phosphorus ions. The different behavior of lactate production in normal and leukaemic cells according to the concentration of inorganic phosphate is clearly evidenced in fig 1 and table II. The statistical

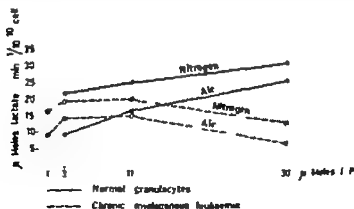


Fig 1 Lactate production of normal and leukaemic leukocytes in a nitrogen or air atmosphere.

Table II

Percentage of Pasteur effect and significance of the difference of the averages in the presence of varying concentrations of inorganic phosphate in normal and leukaemic leukocytes.

Inorganic phosphate mM	1		3		11		30	
	Pasteur Effect %	P	Pasteur Effect %	P	Pasteur Effect %	P	Pasteur Effect %	P
Normal leukocytes			61.0	0.0025	36.0	0.005	21.0	0.025
C. M. L.	42.0	0.0025	30.3	0.05	35.0	0.0125	38.5	0.012
C. L. L.	70.5	0.0025	56.3	0.0025	70.5	0.0025		
A. L.	30.3	0.1	39.4	0.025	46.0	0.025		

significance of Pasteur effect in normal leukocytes increased as the phosphorus ions decreased in the medium, whereas it remained almost the same in leukaemic leukocytes.

Even the O_2 consumption in the presence of succinate and glycerophosphate was affected by inorganic phosphate in the extracellular medium (table III) The optimal concentration of phosphate in the extracellular medium was, in normal cells, lower than that required for glycolysis (3 μMol/ml instead of 30 μMol/ml) An increase in the amount of phosphates exceeding 3 μMol inhibited oxygen consumption.

Table III

O_2 uptake of normal and leukaemic leukocytes (μMol O /min/10^{10} cells) in the presence of succinate and glycerophosphate as well as of varying inorganic phosphate concentrations.

Substrates		20mM Succinate	20mM Succinate + 3mM P	20mM Succinate 30mM I.P	6mM Glycero-phosphate	6mM Glycero-phosphate + 30mM I.P
Normal cells	M	5.07	3.45	4.08	4.12	3.86
	σm	0.61	0.13	0.44	0.35	0.54
C. M. L.	M	2.83	3.50		3.20	2.79
	σm	0.84	0.36		0.41	0.39
A. L.	M	3.75	3.77		3.55	3.67
	σm	0.91	0.84		0.8[illegible]	0.80

The leukocytes of chronic myelogenous and acute leukaemia, which under all our experimental conditions took up less oxygen than the normal leukocytes, appeared to be less affected by the extracellular phosphate concentration. The slight increase in oxygen consumption observed with 3 μMol of phosphate, is not statistically significant.

An endogenous respiration, without exogenous additions to the incubation medium, was observed in both normal and leukaemic leukocytes. Quantitatively the endogenous respiration of granulocytes was markedly higher than that of myeloid leukaemic cells and of blast cells of acute leukaemia. Addition of succinate or glycerophosphate increased cell respiration markedly. Succinate appeared to be more active than glycerophosphate in the absence of artificial electron acceptors (table IV) whereas in the presence of toluidine blue glycerophosphate-oxydase activity was somewhat higher than that of succino-oxydase (table VIII). The addition of glucose, in the presence of 3 μMol of inorganic phosphate, clearly inhibited oxygen consumption in normal cells (table IV).

Table IV

Influence of various substrates on cellular respiration in the presence of optimal concentration of inorganic phosphat (3 mM)

Substrates	Endogenous substrates	Id. + 20mM Succinate	Id. + 10mM Glycero-phosphate	Id. + 10mM Glucose
Normal cells	4.02 ± 0.60	3.45 ± 0.13	4.12 ± 0.35	2.67 ± 0.31
C. M. L.	2.67	3.50 ± 0.36	3.20 ± 0.41	
A. L.	3.24	3.77 ± 0.84	3.55 ± 0.82	

As this finding is controversial, we have controlled the influence of glucose under different experimental conditions, in both normal and leukaemic cells. The results are summarized in table V. There is clear evidence that glucose addition, either alone or combined with phosphate and/or different substrates, always decreased leukocyte oxygen consumption (positive Crabtree effect). Such behavior was observed in both normal and leukaemic cells. The significance of this finding has always been sufficient and, under certain experimental conditions, even outstanding.

Since in our cell preparations the presence of both positive Pasteur and Crabtree effect seems to indicate a reciprocal interference between respiration and glycolysis, lactate production and O_2 consumption respectively in the absence and presence of 2-4-dinitrophenol (DNP) and iodoacetic acid (IAA) were studied (table VI). The addition of DNP to the incubation medium completely reversed the Pasteur effect which was observed in normal cells in the presence of a relative deficiency of IP (3 μMol/ml). On the other hand oxygen consumption was always decreased by glucose

Table V

…ose influence (10 µMol/ml) on the O uptake in normal and leukaemic leukocytes (µMol O_2/min…
Mean values ± σm.

	Normal cells		C.M.L.		A.L…
	I. U./10^{10} cells	% Crabtree effect	I. U./10^{10} cells	% Crabtree effect	I. U./10^{10} cells
…ogenous substrates	3.37±0.41	25	2.64±0.19	[illegible]	2.63±0.44
+ 10 mM Glucose	2.71±0.38		2.15±0.22		2.61±0.56
…ogenous substrates + 3 mM I. P	4.02±0.60	34			
+ 10 mM Glucose	2.67±0.31				
…ogenous substrates + 30 mM I. P	3.34±0.44	29	2.48±0.28	29	2.96±0.58
+ 10 mM Glucose	2.38±0.55		1.77±0.17		2.12±0.22
…M Succinate	4.83±0.40	33			
+ 10 mM Glucose	3.23±0.54				
…M Succinate + 3 mM I. P	5.45±0.13	31	3.50±0.36	28	3.77±0.84
+ 10 mM Glucose	3.81±0.31		2.55±0.33		3.50±0.82
…M Succinate + 30 mM I. P	4.05±0.72	31			
+ 10 mM Glucose	2.81±0.29				
…M Glycerophosphate + 30 mM I. P	3.86±0.54	38	2.79±0.29	23	3.67±0.80
+ 10 mM Glucose	2.40±0.10		2.16±0.31		7.12±0.96

Table VI

Influence of DNP and IAA on glycolysis and respiration in normal leukocytes.

Gas phase	10mM Glucose	30mM Succinate	0.01 mM DNP	0.1 mM IAA	Lactate production µMol/min/10^{10} cells	O_2 uptake µMol/min/10^{10} cells
Nitrogen	+	—	—	—	23.03±2.55	[illegible]
Air	+	—	—	—	8.66±1.55	2.67±0.31
Air	+	—	+	—	22.4	
Air	—	—	—	—		4.02±0.60
Air	—	+	—	—		5.43±0.13
Air	+	+	—	—		3.81±0.31
Air	+	+	—	+		3.55
Air	—	+	—	+		3.80

addition, except in those preparations where IAA had also been added.

Trying to clarify the mechanism through which phosphate deficiency slowed down glycolysis, we examined the behavior of triosephosphate esters (glyceraldehyde 3-P and dihydroxyacetone-phosphate). Since the glyceraldehyde-3-P-dehydrogenase reaction utilizes directly inorganic phosphate, a shortage of phosphorus ions could have resultet in an accumulation of triosephosphate esters, namely in the presence of ATP. For such determinations we utilized cell homogenates and not intact cells to favor ATP consumption.

Table VII

Glyceraldehyde-3-phosphate (GAP) and dihydroxyacetonephosphate (DOAP) production (μMol/min/10^{9} cells) in normal leukocyte homogenates with or without adding ATP 0.1 mM and DNP 0.1 mM.

Gas phase	I. P	DNP	ATP	GAP + DOAP
Nitrogen	—	—	—	0.536
Air	—	—	—	0.440
Air	—	+	—	0.466
Air	—	—	+	0.524
Air	3 mM	—	+	0.11

From table VII it appears that the quantity of triosephosphate esters accumulated as a result of phosphate shortage, was definitely lower than the lactate amount which was not produced under the same experimental conditions. In the presence of inorganic phosphate, we found the lowest accumulation of these intermediates. By adding ATP the amount of glyceraldehyde-3-phosphate and dihydroxyacetonephosphate produced was 5 times greater. DNP did not markedly affect the accumulation of triosephosphate esters.

The activities of the glycolytic and respiratory enzymes were determined in cell lysates (table VIII). The table includes the normal cases which we already mentioned and a series of leukaemic patients which enabled us to calculate reliable averages for each group. In order to make the cases of chronic myeloid leukaemia as much homogenous as possible, they were divided into two groups.

The hexokinase activity was the lowest of all other enzyme activities in both normal and leukaemic cells. It was very low in blast cells of acute leukaemia. In myeloid leukaemia the activity of this enzyme proved to be inversely related to the percentage of blast cells. The antiblastic treatment favored the appearance of a cellular strain characterized by a higher hexokinase content, almost equal to that of normal cells. In one case, where a complete haematologic remission was obtained, hexokinase activity was found to be 19 units per 10^{10} leukocytes. No other glycolytic enzyme showed a similar behavior.

The fructose-6-P kinase and aldolase were the rate-limiting enzymes after hexokinase; they were more active in chronic myeloid leukaemia than in normal cells. In all cell types the glucose-6-P dehydrogenase showed an activity 10 times higher than that of hexokinase. Respiratory enzymes were found to be present in a slightly lower amount.

Table VIII

…vity of some enzymes of glycolysis, of pentose shunt and of respiration in normal and leukaemic…
(μMol substrate/min/10^{10} cells)

…zymes	Normal cells	C.M.L. acute phase	C.M.L. under therapy	C.L.L.	A.L.
…okinase	11.22 ± 1.2	5.11 ± 1.2	8.13 ± 1	4.2 ± 1 4	5.3 ± 0 7
…-P kinase	13.5 ± 1.5	27.3 ± 5.7	24.00 ± 3.6	8 5 ± 2.3	19 4 ± 6.7
…P aldolase	14 1 ± 1 0	30.0 ± 5.9	26.00 ± 4.7	6.0 ± 1.6	25.0 ± 5.1
…ose-isomerase	1438 ± 237		998	448*	
…-dehydrogenase		traces	7.5**		traces
…P-dehydrogenase	346 ± 21	418 ± 76	436 ± 34	108 ± 28	224 ± 40
…-kinase	616 ± 23	783 ± 134	770 ± 61	214 ± 52	512 ± 88
…lase	206 ± 24	187 ± 35	306 ± 26	57 ± 30	104 ± 21
…uvate-kinase	296 ± 20	396 ± 73	417 ± 34	31 7 ± 5	214 ± 57
…tate-dehydrogenase	300 ± 45	337 ± 57	372 ± 37	133 ± 43	346 ± 99
…-P-dehydrogenase	109 ± 11 1	56 ± 9	86 ± 9	24 ± 6	47 ± 10
…late-dehydrogenase			124 ± 11	133 ± 31	
…chrome-oxydase	4.07 ± 0.9		1 11 ± 0.19	1.0 ± 0.31	4.78 ± 0.7
…-oxydase	6.4 ± 0.6		3.04 ± 0.34		2.01 ± 0.6

…e case **mean values of 4 cases.

Table IX

Activity of some enzymes (μMol/min/mg protein) in cellular fractions.

		Hexokinase	Lactate dehydrogenase	Malate dehydrogenase	Succinate dehydrogenase
Normal cells	SN 1	29.0	228.3	175.0	[illegible]
	PR 1				
	SN 2	12.9	235 7	57.0	16.4
	PR 2*	7.2	31 4	6.1	15.1
C. L. L. 1	Cytolysate	24.6	146 7	546.7	
	SN 1	20 0	133 3	916.7	7.3
	SN 2*	12.4	108.3	775 0	
	PR 2	2.6	14 3	171 7	28.4
C. L. L. 2*	Cytolysate	18.9	183 2	127.5	
	SN 1	13 0	131.5	83 7	
	SN 2*	10.5	235 3	134 5	
	PR 2*	3.7	29 9	20.9	

Table X

O_2 uptake (μMol/min/mg protein) of some cellular fractions.

	SN 1	SN 2*	PR 2*
Normal cells 1	21.2	16.7	17.2
Normal cells 2*		2.2	5.9
C. L. L. 1	6 1	6.7	32.0
C. L. L. 2*	34 7		33.5

Hexokinase activity of cytolysate expressed on triose equivalents appeared slightly lower than the amount of lactate formed.

Some preliminary investigations were carried out on small number of subjects to define the cell fraction containing the enzymatic activities. The temporary results are gathered in tables IX and X. Whereas lactic acid dehydrogenase was present almost entirely on soluble cytoplasmatic fractions, part of the hexokinase activity occurred in precipitate fractions the succinate dehydrogenase activity and the respiratory activity in SN I and PR 2. The technique should be further defined in order to get a higher degree of certainty in attributing the enzymatic and respiratory activity to single cellular fractions.

Discussion and Conclusions

Some factors controlling the rate of glycolytic pathway are known thanks to the basic studies of Beck and Valentine (3 4) and the subsequent researches of Loehr and Waller (27) of Grignani and Lohr (21) and Grignani et al. (20) Hexokinase seems to be the rate-limiting factor of the glycolysis of normal and particularly of leukaemic leukocytes. We were able to convalidate the pacemaker role played by hexokinase. However activity of this enzyme is not sufficient to allow the lactate production we could observe. This may be related either to glycogenolysis which is likely to occur under our experimental conditions or to the fact that part of hexokinase is bound to cell structures and is not assayed among the soluble cytoplasmic enzymes. This hypothesis is supported by our results.

The hexokinase is likely to be connected with stromal and possibly mitochondrial fractions accordingly to what was observed in ascites tumor cells (36) Hexokinase activity bound to cell structures deserves to be further investigated

The hexokinase anyway is not the only factor capable of affecting glycolysis rate. Beck (2) reported that even ADP deficiency due to the insufficient hexokinase activity played an important role in regulating cell glycolysis of acute leukaemia. Inorganic phosphate also has an important part which was found to be outstanding in normal cells. This finding is similar to that noticed by Wu and Racker (36) in ascites tumor cells. The endeavour to explain influence of phosphate by blocking glyceraldehyde-3-P dehydrogenase was only partly positive since it was not observed an accumulation of dihydroxyacetonephosphate and glyceraldehyde-3-phosphate quantitatively comparable to the decreased lactate production

The importance of inorganic phosphate is shown namely in aerobic conditions, presumably in the presence of active respiration. Since uncoupling agents, such as DNP prevented the decrease of the glycolysis rate under aerobic conditions, and the addition of glucose inhibited the oxygen uptake, it is likely that glycolysis and respiration are competitively connected in white blood cells.

The presence in leukocytes of a positive Crabtree effect is not unanimously accepted. Although confirmed by Estes et al. (15) it was denied by Beck (2) and by Loehr and Waller (27) In our studies the Crabtree effect was always found, not only when glycolysis was inhibited by IAA. It is possible that active glycolysis in vitro may compete with respiration in utilizing some metabolites (ADP for instance) and inorganic phosphate

Inorganic phosphate is important also for respiration but the optimal phosphate amount was lower than that required for glycolysis, even when the addition of appropriate substrates (succinate, glycerophosphate) had activated markedly oxydative processes. Oxygen consumption, on the other hand is generally lower in leukocytes than in other cells, and the glycolysis-respiration ratio is in favor of glycolysis (7 3)

Besides, though this result is to be confirmed, it seems that leukocyte glycolysis represents the basic metabolic pathway for energetic aims. In fact, glycolysis inhibitors block phagocytic activity of leukocytes, whereas cyanide and DNP have no effect (23, 18) This is confirmed by Chernyak s investigations on P^{32} incorporation in intracellular ATP (13) and by the lack of oxydative phosphorylation in intact granulocytes (14) Our studies confirmed also that succinate oxydase, which is a basic step of the Krebs cycle, is characterized by an activity lower than that of all glycolysis enzymes, including hexokinase.

Our experiments did not show qualitative differences in the energy metabolism of normal and leukaemic leukocytes. So far moreover only the absence of cytoplasmic glycerophosphate dehydrogenase was reported in acute leukaemia (27) Even from our investigations some significant quantitative differences emerged concerning both glycolysis and O_2 uptake. The production of lactate and the O_2 consumption are lower in leukaemic cells, although in chronic myeloid leukaemia they are almost equal to granulocyte values, as there is a progressive haematologic improvement due to

therapy. As to glycolysis, this phenomenon seems to be supported by the increase of hexokinase with the disappearance of blast cells.

Summary

In white blood cells, inorganic phosphate increased the glycolysis. White blood cells showed evident Pasteur and Crabtree effects. In normal leukocytes the Pasteur effect was partly corrected by optimal concentrations of inorganic phosphate and completely reversed by dinitrophenol. Maximal stimulation of respiration was obtained in the presence of phosphate and succinate. All glycolytic enzymes were present in our preparations. Hexokinase showed the lowest activity. An amount of this enzyme was detected bound to the cell structures and is to be further investigated.

Résumé

Les phosphates anorganiques augmentent la glycolyse dans les leucocytes. Ceux-ci montrent des effets de Pasteur et de Crabtree évidents. Dans les leucocytes normaux, l'effet de Pasteur est en partie corrigé par une concentration optimale de phosphates anorganiques et complètement inversé par du dinitrophénol. Une stimulation maximale de la respiration a été obtenue en présence de phosphates et de succinates. Tous les enzymes glycolytiques étaient présents dans nos préparations. L'hexokinase montra l'activité la plus faible. Une partie de cet enzyme se trouva être fixée aux structures cellulaires et sera l'objet d'études postérieures.

Zusammenfassung

Anorganisches Phosphat bewirkt in Leukozyten eine Zunahme von Glykolyse und Atmung. Weisse Blutzellen zeigen deutliche Pasteur- und Crabtree-Effekte. In normalen Leukozyten wird der Pasteureffekt durch optimale Konzentrationen von anorganischem Phosphat zum Teil korrigiert und durch Dinitrophenol vollständig umgekehrt. Bei Gegenwart von Phosphat und Succinat ergibt sich eine maximale Stimulation der Respiration. In den Präparaten waren alle glykolytischen Enzyme vorhanden. Hexokinase zeigte die geringste Aktivität. Eine bestimmte Menge dieses Enzymes ist an Zellstrukturen gebunden und soll weiterhin untersucht werden.

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Über den Nachweis einer sauren Lipase in weißen Blutzellen

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Obwohl die weißen Blutzellen eine wichtige Stellung im Fettstoffwechsel einnehmen, liegen bis jetzt nur wenige Mitteilungen über eine lipolytische Aktivität in diesen Zellen vor. Izak und de Vries (7) inkubierten Leukozyten in Plasma mit einer Olivenölemulsion bzw. Chylomikronensuspension und konnten eine Zunahme der freien Fettsäuren in den inkubierten Zellen finden. Sie schlossen daraus auf eine intrazelluläre Hydrolyse des phagozytierten Fettes. Hardin et al. (5) untersuchten die Spaltung von β-Naphthollaureat durch Granulozyten und Lymphozyten; dieses Substrat kann aber auch von unspezifischen Esterasen gespalten werden. Wir haben kürzlich eine Lipaseaktivität in Granulozyten, Lymphozyten und Makrophagen im alkalischen Bereich beschrieben (1, 2).

Da während der Phagozytose das intrazelluläre pH in Leukozyten nach der sauren Seite umschlägt (9) und das phagozytierte Material zu einem beträchtlichen Teil aus Lipiden besteht, wäre eine lipolytische Aktivität im sauren Bereich von großer physiologischer Bedeutung. In der folgenden Arbeit wird aus diesem Grunde die lipolytische Aktivität in verschiedenen weißen Blutzellen im sauren Bereich untersucht.

Methodik

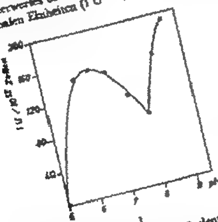
Enzym. Die Leukozyten wurden aus peripherem Blut mittels Dextran isoliert (Einzelheiten s. 1). Die isolierten Zellen wurden dreimal eingefroren und aufgetaut und hierauf mit einem motorbetriebenen Glashomogenisator nach Potter-Elvehjem im Eisbad homogenisiert.

Die untersuchten Zellen stammten von Patienten, die eine ausgeprägte myeloische Reaktion aufwiesen oder an chronischer myeloischer bzw. chronischer lymphatischer Leukämie erkrankt waren.

Substrat Als Substrat diente eine Emulsion von Kokosfett in Plasma, die mit einem Ultra-Turrax in einer Konzentration von 30% hergestellt wurde. Diese Emulsion erwies sich als sehr stabil, wurde jedoch für jeden Versuch frisch bereitet.

Messung der lipolytischen Aktivität. Versuchsansatz (Endkonzentration): Kokosfett 6%, Albumin 4% (Bovine albumin powder Fraction V Armour Pharmaceutical Co., Kankakee, Ill., U.S.A.) Glucose 0,015 m, Phosphatpuffer 0,006 m. Das pH des Inkubationsgemisches wurde durch tropfenweises Zugeben von n/10 NaOH bzw HCl eingestellt. Das Enzym wurde in Form des Homogenates entsprechend 4×10^7 bis 5×10^8 Zellen pro Ansatz zugesetzt. Das Gesamtvolumen betrug 5,0 ml.

Der Ansatz wurde im Wasserbad bei 37 °C unter Stickstoff geschüttelt. 5 Minuten nach Inkubationsbeginn wurden 2,0 ml entnommen (Start der Reaktion) und darin die freien Fettsäuren nach Dole und Meinertz (3) gemessen. Nach weiteren 30 Minuten wurden wieder in 2,0 ml die freien Fettsäuren bestimmt. Beim Leerwert wurde 0,9%ige NaCl an Stelle der Fettemulsion zugesetzt. Die Spontanhydrolyse der Triglyceride wurde im zellfreien Ansatz erfaßt. Unter den gegebenen Bedingungen verlief die Reaktion über einen Zeitlauf von über 60 Minuten linear. Die lipolytische Aktivität war der zugesetzten Enzymmenge proportional. Die lipolytische Aktivität wurde aus der Differenz der freien Fettsäuren vor und nach Inkubation unter Berücksichtigung des Leerwertes und der Spontanhydrolyse berechnet. Die Aktivitäten werden in Internationalen Einheiten (I U = µMol freie Fettsäuren/Min./10^{12} Zellen) angegeben.



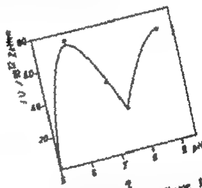


Abb 1 pH Kurve Zellen von Patienten mit myeloischer Reaktion. Ordinate: Lipaseaktivität in internationalen Einheiten. Die eingetragenen Werte sind Mittelwerte von 2 Patienten. Es findet sich ein pH-Optimum im sauren und alkalischen Bereich.

Abb 2. pH Kurve Zellen von Patienten mit chronischer Myelose. Ordinate: Lipaseaktivität in internationalen Einheiten. Die eingetragenen Werte sind Mittelwerte von 2 Patienten. Es findet sich ein pH-Optimum im sauren und alkalischen Bereich.

Ergebnisse

pH Kurve Abb 1, 2 und 3 zeigen die pH Kurve für Zellen von myeloischer Reaktion, chronischer myeloischer Leukämie bzw chronischer lymphatischer Leukämie. Wie aus den Abbildungen ersichtlich, findet sich bei der myeloischen Reaktion (Abb. 1 bei der chronischen myeloischen Leukämie (Abb 2) eine

lytische Aktivität im alkalischen und im sauren Bereich. Das pH Optimum im alkalischen Bereich liegt über 9 im sauren Bereich zwischen 6.0 und 6.5 Im Gegensatz dazu zeigen Zellen von chronischer lymphatischer Leukämie nur eine alkalische Lipaseaktivität mit einem pH-Optimum über 9 Im sauren Bereich konnte bei diesen Zellen keine lipolytische Aktivität nachgewiesen werden (Abb 3)

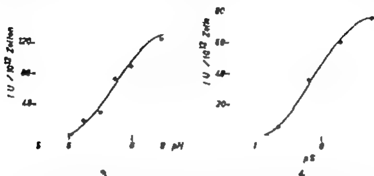


Abb. 3. pH-Kurve. Zellen von Patienten mit chronischer Lymphadenose. Ordinate Lipaseaktivität in internationalen Einheiten. Die eingetragenen Werte sind Mittelwerte von 4 Patienten. Es findet sich nur im alkalischen Bereich ein pH-Optimum.

Abb. 4. pS-Kurve. Abszisse negativer Logarithmus der Substratkonzentration (pS) in g%. Ordinate. Lipaseaktivität pro 10^{12} Zellen.

pS-Kurve Abb 4 zeigt die lipolytische Aktivität bei einem pH von 6.0 für Zellen von Patienten mit myeloischer Reaktion bei verschiedenen Substratkonzentrationen. Wie aus dieser Abbildung hervorgeht, liegt das Substratoptimum bei einer Konzentration von 6 g%

Bei Gültigkeit der Gleichung

$$v = \frac{V\,(S)}{K_s + (S)}$$

nach Michaelis und Menten (8) erhält man eine Gerade, wenn man v gegen $\frac{v}{(S)}$ aufträgt. Der Achsenabschnitt auf der Ordinate entspricht dann der maximalen Geschwindigkeit V und der Achsenabschnitt auf der Abszisse entspricht $\frac{V}{K}$ (6) (Abb 5) Nach dieser Methode wurde für unsere Versuchsanordnung eine Michaelis-Konstante von 1.14×10^{-2} m für die saure Lipase berechnet (über

die Anwendbarkeit dieser fermentkinetischen Gesetze s. 10 11) Dieser Wert entspricht größenordnungsmäßig der MICHAELIS-Konstante, die wir für die Lipase im alkalischen Bereich gefunden haben (1)

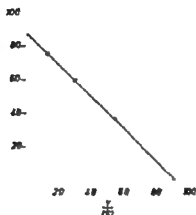


Abb. 5. Graphische Ermittlung der MICHAELIS-Konstante (6).

Diskussion

In der vorliegenden Untersuchung konnte eine Lipase im sauren Bereich in den Granulozyten bei myeloischer Reaktion und chronischer Myelose nachgewiesen werden. Dies ist insofern von besonderem Interesse, als diese Zellen im Gegensatz zu Lymphozyten die Fähigkeit zur Phagozytose besitzen. Es ist bekannt, daß das intrazelluläre pH während der Phagozytose in den sauren Bereich umschlägt. Eine Aufspaltung von Triglyceriden während der Phagozytose ist daher nur bei Vorhandensein einer im sauren Bereich wirksamen Lipase möglich.

Unsere Untersuchungen stehen im Einklang mit den Ergebnissen von ELSBACH UND RIZACK (4) die im Homogenat von Exsudatzellen aus der Peritonealhöhle von Kaninchen ebenfalls eine saure Lipase nachweisen konnten. Auch diese Zellen sind zur Phagozytose befähigt.

Da es während der Phagozytose zu einer Steigerung des Lipidumsatzes kommt (12) ist anzunehmen, daß bei Absinken des intrazellulären pH die beschriebene Lipase aktiviert wird und dadurch vermehrt Fettsäuren und Glycerin zur Energiegewinnung verfügbar werden.

Der zusätzliche Nachweis einer sauren Lipase in den Granulozyten stellt ein weiteres biochemisches Unterscheidungskriterium gegenüber den Lymphozyten dar die nur eine alkalische Lipase aufweisen. In weiteren Untersuchungen soll abgeklärt werden, in welchem Stadium der Zellreifung diese saure Lipase auftritt und ob sie sich auch in den entdifferenzierten Zellen bei akuter Myelose nachweisen läßt.

Zusammenfassung

Neben einer alkalischen Lipaseaktivität wird in Granulozyten von Patienten mit myeloischer Reaktion und chronischer Myelose auch eine Lipolyse im sauren pH Bereich nachgewiesen. Für diese Lipase liegt das pH-Optimum zwischen 6,0 und 6,5, das Substratoptimum liegt bei Verwendung von Kokosfett in Form einer Emulsion im Plasma bei 6 g%, die MICHAELIS-Konstante beträgt $1{,}14 \times 10^{-4}$ m. Im Gegensatz dazu wird in den Leukozyten von Patienten mit chronischer Lymphadenose nur im alkalischen Bereich eine Lipaseaktivität gefunden. Die mögliche physiologische Bedeutung einer sauren Lipase im Zusammenhang mit der Phagozytose wird diskutiert.

Summary

In granulocytes of patients with chronic myelosis presenting a myeloid reaction, lipolysis can be demonstrated in the acid pH range, as well as alkaline lipase activity. The optimum pH for this lipase lies between 6.0 and 6.5, and the optimum substrate is obtained with 6 g% coconut oil emulsion in plasma. MICHAELIS' constant is 1.14×10^{-4} M. In contrast, lipase activity is found only in the alkaline range in the leukocytes of patients with chronic lymphadenitis. The physiological significance of acid lipase in connection with phagocytosis is discussed.

Résumé

Dans les granulocytes de malades ayant une réaction myéloïde ou une myélose chronique, une lipolyse en milieu acide est mise en évidence à côté d'une activité de lipase alcaline. L'optimum du pH pour cette lipase se trouve entre 6,0 et 6,5. La concentration optimale d'une émulsion de graisse de noix de coco employée comme substrat est de 6 g%, la constante de MICHAELIS de $1{,}14 \times 10^{-4}$. Dans les granulocytes de malades ayant une lymphadénose chronique, on ne trouve une activité de lipase qu'en milieu alcalin. L'importance physiologique possible d'une lipase acide dans la phagocytose est discutée.

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Thrombocytic and Thrombopenic Factors in Normal Human Serum*

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Ruth A. Martin and Albert A. Dietz

The subject of humoral regulation of thrombocytes has reached, as any new concept, the phase of confusion in its march to eventual clarification. Yet, the existence of humoral control of thrombopoiesis has been accepted by several investigators working independently in different laboratories and using varying methods of approach (1 to 12) The state of confusion is illustrated by a variety of experimental conditions and results (1 2, 4 to 12)

A previous communication indicated the existence of a factor which stimulated the production of megakaryocytes without increasing thrombocytes. This report is correlated with the present one to suggest the presence of two distinct factors, one for megakaryocyte and another for thrombocyte control.

Methods and Materials

Normal human blood was obtained from blood banks. It was collected in ACD anticoagulant solution. The age of the blood varied from 2 to 60 days. The plasma was siphoned off after centrifugation. Several batches of plasma were pooled to make one liter The plasma was dialyzed against running cold tap water for 48 to [illegible] hours.

The serum was fractionated with 55% ammonium sulfate and the albumin fraction was separated on the continuous flow paper curtain electrophoresis. Zinc acetate in 1 M concentration was added to the albumin solution for an initial concentration of 0.0012 M (Zn 1) after adjustment to pH of 6.1 with the precipitate usually negligible. T the supernatant was added 1 M zinc acetate for a concentration of 0.002025 (Zn 2). The procedure was repeated after each centrifugation to obtain the following concentrations 0.00385 M (Zn 3), 0 00614 M (Zn 4), 0.0136 (Zn 5), and to the final supernatant was added saturated solution of ammonium sulfate with resultant precipitate labeled Zn 6.

Biological testing was done on total of 211 New Zealand white rabbits of either sex, approximately 4 months of age. Because of relative large volumes of fluid, the rabbits

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were injected intravenously 3 times daily on 2 successive days with doses equivalent to 4 4 ml of original serum per lb. of body weight. Blood studies were done prior to injections and included thrombocyte, erythrocyte and leukocyte counts, hemoglobin and microhematocrit determinations, reticulocyte and differential counts of stained blood preparations. The blood studies were repeated after injections twice daily At termination of studies, the animals were killed. Bone marrow spleen, liver lymph nodes and lungs were examined histologically

Fresh normal serum was injected intravenously into 15 rabbits, (2250 g) to determine the effect on the thrombocyte pattern of the peripheral blood. Zinc acetate fractions from Zn 3 to 6 inclusive were injected intravenously into 120 rabbits. Multiple injections of thrombocyte increasing and decreasing fractions were administered over periods of 42 to 66 days to 8 rabbits. Egg albumin in 10% quantities were injected intravenously into 10 rabbits. All animals were 4 months of age and weighed approximately 5 lbs. each.

Results

Chemical determinations of the protein content showed reduction from 6.6 g per 100 ml of the original serum to 0.46 g for the thrombopoietic fraction and 0 22 g for the thrombopenic (table 1) The protein content of the fractions varied with the technique in preparing each batch of serum. While paper electrophoresis of the thrombopoietic fraction showed albumin only starch-gel electrophoresis revealed an increase in the highly mobile acidic alpha glycoprotein and pre-albumin, with presence of some bulk albumin, post-albumin and alpha$_2$-globulin.

Table I

Protein content of serum and its fractions at succeeding steps of fractionation for substances which increased and decreased thrombocytes in the peripheral blood of rabbits.

Nature of Material	Protein content in grams percent of: Thrombocyte increasing substance	Thrombocyte decreasing substance
Original Serum	6.6	6 6
0 to 55% ammonium sulfate fraction	3.9	3.9
Albumin obtained by continuous flow electrophoresis of 0 to 55% ammonium sulfate fraction	1.2	1.2
Zinc acetate fractions which produced a significant increase or decrease of thrombocytes	0.46	0.22

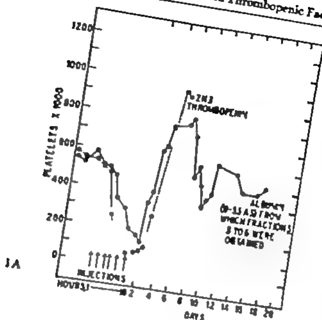
Paper electrophoretic patterns of the thrombopenia inducing Zinc Acetate fraction showed 13 9 / albumin, 2.8 / alpha$_1$-globulin, 69 4 / alpha$_2$-globulin and 13 9 / of beta globulin. Starch-gel

preparations followed closely this composition. In a few batches of serum, the thrombopenic fraction was composed predominantly of albumin on paper electrophoresis, but on starch-gel electrophoresis there was an admixture of $alpha_1$-globulins

The significant Zinc Acetate fractions referred to above are Zn 3 4 5 and 6 respectively In most preparations, Zn 3 fraction produced significant thrombopenia. Of 17 pooled batches of serum, the thrombopenic factor was found in 11 out of 20 rabbits and 6 batches of serum showed no effects. The thrombopenic factor gradually reduced thrombocytes by 80 / in 24 hours. On the third day the thrombocytes were normal For the next 3 days the rebound was 50 / of normal (fig 1A) Rabbits with repeated injections of thrombopenic factor for 42 days maintained a thrombopenia for that period (fig 1B) Total albumin, removed from 0 to 55 ammonium sulfate fraction, paralleled Zn 3 fraction in its thrombopenic effect and suggests the factor to be more potent than the thrombocytic component (fig 1A)

Fresh serum given to 12 rabbits, showed a marked decrease of thrombocytes within 15 minutes, a rise in 45 minutes, and a return to normal in 6 to 8 hours. The rebound was absent in most rabbits (fig 2) Tissues showed thrombocytes in liver lungs and spleen and adhesion to vascular endothelium these findings suggest that this type of thrombopenia was due to injury to thrombocytes. With Zn 3 thrombopenic factor the decrease was gradual, the low peak was in 24 hours and thrombopenia lasted for 3 days. Rebounds were constant. There was no retention of thrombocytes in organs. Granulocytes were increased 4-fold. Erythrocytes were normal. It is assumed that the thrombopenic factor inhibited thrombocyte production and the gradual reduction was consistent with physiological disintegration and failure of replacement.

In 17 pooled batches of serum, Zn 6 fraction produced a characteristic pattern of thrombocyte increase in 8 batches, Zn 5 fraction in 3 and in 4 batches the increasing factor was distributed in Zn fractions 4 and 5 Two batches failed to show any change. Preparation of Zinc Acetate fractions presented technical problems which failed to give constant results as seen in variations of protein and their zones on paper and starch-gel electrophoresis. Of 90 rabbits injected with fractions Zn 4 5 or 6 thrombocytosis occurred in 42 with Zn 6 in 24 with Zn 5 in 8 with Zn 5 and 4 in 11 there was no response.


PLATELETS X 1000
1200
1000
800
600
400
200
0
THROMBOPENIN
WHICH FRACTIONS
3 TO 6 WERE
OBTAINED
INJECTIONS
HOURS
2 4 6 8 10 12 14 16 18 20
DAYS

1A

1B

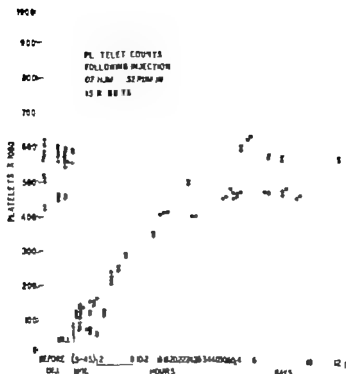


Fig 2. Thrombocyte counts in 12 rabbits injected intravenously with fresh normal human serum for comparison with patterns in fig 1A and B.

The pattern of thrombocytosis showed an initial rise in 2 days, peaks on fourth, sixth, seventh and eleventh days and thrombocytosis for 16 to 30 days with smaller peaks. Multiple injections sustained thrombocytosis for 66 days, the period of experiment. Neither erythrocytes nor leukocytes showed numerical changes. Exposure of *thrombopenic and thrombocytic factors to heat showed retention* of activity with 60° C heat, and destruction with boiling

Daily passive transfer of serum from donor rabbits injected with thrombocytic fraction, Zn 6 into recipient rabbits showed in the

Fig 1A and B A Example of thrombocyte counts after 6 injections in 48 hours of thrombopenic fraction Zn 3 and of albumin (solid line). Decrease was gradual and lasted for 3 days. Albumin from which 6 fractions were obtained showed decrease. The thrombopenic component is more potent than thrombocytic. B Example of thrombocyte counts after repeated injections of thrombopenic factor Thrombopenia lasted for 42 days.

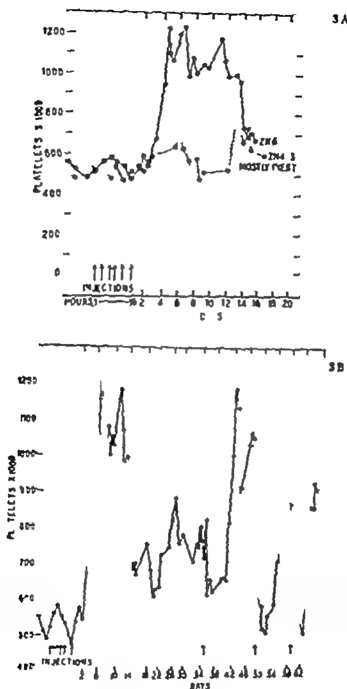


Fig. 3A and B. A. Example of repetitive thrombocyte counts with thrombocytic fraction Zn 6. Broken line represents thrombocyte counts of inert fractions. B. Example of multiple injections of thrombocytic fraction, Zn 6, with thrombocytosis for 62 days.

first days little or no thrombocytosis. The injected thrombocytic factor in donor animals had been removed from the circulation with in 24 hours. On fifth and sixth days, the recipient rabbits showed a thrombocytosis for 8 to 16 days (fig 4) The appearance of the fac tor on the fifth day suggests its production by the donor animal. Passive transfer of thrombopenic factor showed similar results.

Examination of bone marrow of rabbits injected with thrombocytic and thrombopenic factors did not show significant changes in the number of megakaryocytes. There were no foci of hyper plasia of megakaryocytes nor an increase of megakaryoblasts.

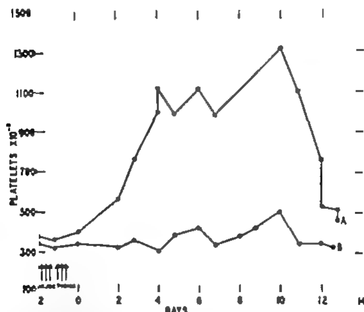


Fig 4 Example of thrombocyte counts after serum injections on 7 successive days from donor rabbits given thrombopoietic fraction, Zn 6. «A» is the thrombocyte pattern of recipient rabbit with serum from donor rabbit given thrombocytic factor 5 days previously 'B' is the pattern of recipient rabbit with serum of donor rabbit injected 7 days previously with thrombocytic factor

Discussion

One of the significant problems in thrombopoiesis involves the conflicting reports on thermostability of the thrombocytic factor Linton (10 12) Schulman et al. (6) acidified and boiled plasma for 30 minutes, giving the filtrate by mouth daily to

thrombopoiesis, whereas STEINBERG et al. (5) injected a fraction of normal plasma intravenously for 48 hours for continued thrombocytosis and found it thermolabile to 90 C for 1 minute. ODELL AND McDONALD (13) and YAMAMOTO (2) reported the activity of normal (13) and thrombocythaemic serum (2) destroyed or 'less potent' after 100 C for 1 minute or boiling There are two possible explanations for the discrepancies. There may be two thrombocytic factors. Acceptable criteria for significant thrombocytosis vary with each investigation. LINTON's patterns differ from those in this paper

Another problem resides in the significance of subcutaneous injection of several 'non-specific' substances, such as egg albumin, powdered glass and turpentine, which produced thrombocytosis. ODELL s (13) SPECTOR s (8) and our studies of passive transfer of the thrombocytic factor and the appearance of the thrombocytic intrinsic factor upon stimulation should help to clarify the role of non-specific materials. Transfer of serum from donor rabbits injected with 'non-specific substances' have shown presence of intrinsic thrombocytosis factor on the 5th day as with thrombocytic factor of serum. Since injection of egg albumin intravenously did not produce thrombocytosis, the concept of tissue damage and demand for thrombocytes appears to be a plausable explanation.

The presentation of two thrombopoietic factors, one inducing thrombopenia and the other thrombocytosis, re-emphasizes the concept of a pair of antagonistic factors, which under normal conditions maintain a normal balance of thrombocytes (3) In a previous communication, stimulation of megakaryocytes by a fraction of normal human serum was demonstrated (14) The increase was not associated with a corresponding rise of thrombocytes. In the present study neither of the two thrombopoietic factors was associated with significant numerical changes of megakaryocytes. It is proposed that production of thrombocytes and their delivery into the blood stream depend upon two facotrs. One of them produces and matures megakaryocytes, the other stimulates the megakaryocytes to develop thrombocytes.

By employing starch-gel electrophoresis method, it was possible to demonstrate specific proteins in the fractions. Bulk albumin and postalbumin were identified with the thrombocytic factor YAMAMOTO s (2) findings are contrary to ours. He found activity to be concentrated in the beta globulin component as indicated by paper strips after electrophoresis. However our studies agree with those of

Yamamoto in that thrombopoietic activity is concerned with a *macromolecule, protein in nature.*

Summary

Two substances were obtained by fractionation of normal human serum. One fraction increased thrombocytes in the blood of rabbits. Multiple injections sustained thrombocytosis. The second factor decreased thrombocytes in repetitive pattern. Multiple injections sustained thrombopenia. Both substances were thermolabile at 90° C for 1 minute and thermostabile at 60° C for 30 minutes. Passive transfer showed their disappearance from circulation in 24 hours with stimulation of intrinsic thrombopoietic factors on the 5th day. It is postulated that control of normal mechanism of thrombopoiesis includes at least 3 factors. One stimulates megakaryocyte proliferation, another increases thrombocyte production in megakaryocytes. The third, a thrombopenic factor controls the number of thrombocytes for physiological requirements.

Résumé

Par fractionnement de sérum humain normal, deux substances furent obtenues, dont l'une augmente et l'autre diminue le nombre des thrombocytes. L'injection répétée de la première maintient une thrombocytose et celle de la seconde une thrombopénie. Les deux substances sont thermolabiles, exposées à une température de 90 °C pendant une minute, et thermostables, exposées à une température de 60 °C pendant 30 minutes. Après transmission passive, elles disparaissent de la circulation en 24 heures et stimulent les facteurs thrombocytopoïétiques le cinquième jour. Il est supposé que le contrôle du mécanisme normal de la thrombocytopoïèse comprend au moins 3 facteurs. L'un stimule la prolifération des mégacaryocytes, le second augmente la production des thrombocytes dans les mégacaryocytes. Le troisième, un facteur thrombopénique, contrôle le nombre des thrombocytes d'après les besoins physiologiques.

Zusammenfassung

Durch Fraktionierung von normalem menschlichem Serum wurden zwei Substanzen gewonnen. Die eine Fraktion führte zu einem Anstieg der Thrombocyten im Blut von Kaninchen. Wiederholte Injektionen unterhielten die Thrombocytose. Der zweite Faktor ergab eine reproduzierbare Senkung der Plättchenzahl. Multiple Injektionen hielten die Thrombopenie aufrecht. Beide Substanzen waren thermolabil bei 90° C für 1 Minute und thermostabil bei 60° C für 30 Minuten. Bei passiver Übertragung verschwanden die Faktoren aus der Zirkulation innert 24 Stunden mit einer Stimulation endogener thrombopoetischer *Faktoren am 5. Tag. Es wird vermutet, daß die Regulation* des normalen Mechanismus der Thrombopoese mindestens 3 Faktoren erfordert. Der eine stimuliert die Proliferation der Megakaryocyten, der zweite steigert die Plättchenbildung der Megakaryocyten, und der dritte, ein thrombopenischer Faktor reguliert die Thrombocytenzahl nach dem physiologischen Bedarf.

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parenchyma and although they also occur within the lumen of sinusoids, their presence here does not indicate whether they are entering or leaving the marrow

Previous studies of bone marrow recovering from irradiation (7 8) have shown that at a certain stage during the recovery many of its dividing cells (⅓—½ of all telophases) appear to be forming small or medium-sized lymphocytes. Experiments were therefore planned in which the problem of lymphocyte production in guinea pig bone marrow has been further investigated. The results of these experiments are reported in this communication and also characteristic mitoses which are forming small lymphocytes are illustrated in marrow smears. The experiments are in three groups and were designed to answer three questions 1 do lymphocytes still occur in shielded bone marrow after massive irradiation has destroyed these cells in the rest of the body and has also suppressed their production? 2 If lymphocytes do persist in the shielded marrow what proportion, if any are newly formed after the event of irradiation? 3 Are young lymphocytes detectable in bone marrow after it has been isolated from the general circulation? Marrow was labelled with ^{3}H thymidine and was then cultured in the peritoneal cavity of a host whose own lymphocyte levels and production had been grossly suppressed by previous irradiation in the supra lethal range

Material and Method

The experimental animal. Male albino guinea-pigs of the Dunkin-Hartley strain, approximately 400 g weight were used in these studies. Only animals whose RBC and WBC counts were within normal limits were used in the experiments.

Group 1 experiments. A total of 15 animals was studied. In 8 experiments, 720 rads irradiation (the L. D. 100, 18 days) was given to each animal and after 7-day interval the numbers of small and medium-sized lymphocytes (7—10 μ in diameter) were estimated quantitatively in irradiated (humeral) and shielded (femoral) bone marrow in the same animal. I 5 more animals quantitative studies of lymphocytes in shielded marrow were made after the rest of the body had received 1000 rads. I two further animals, the lymphocyte content of shielded marrow was examined after tw days of irradiation to the remainder of the body an initial dose of 1000 rads, followed by further dose of 500 rads on the 4th day To determine the incidence of lymphocytes in the shielded and irradiated marrows, differential counts were made of 4000 nucleated cells in bone marrow smears prepared from each type of marrow Not more than 1000 cells were counted in any one smear

Group 2 experiments. 6 animals were studied in this group. Each was given tritiated thymidine after being lethally irradiated (720 rads) but with shielding of the hind-limbs. To allow daughter cells in the shielded marrow to label adequately liberal interval (48 hours) was allowed between injection of the label and preparation of marrow smears. I one experiment the shielded marrow was examined 3 days after the original

irradiation of the animal in 6 experiments after 4 days and in one experiment after 7 days. The specific activity of the ^{3}H-thymidine was 0.36 per mM in one experiment, 1.41 in two experiments and 1.9 in 5 experiments. The route of injection was intravenous and/or intra-peritoneal. The weight of the animal the time of injection was approximately 400 g. The dosage of ^{3}H-thymidine was 1 μc per g body weight, except in one animal which received 1 μc per 3 g body weight. I 4 animals second injection was given 24 hours after the first dose. The dosage rate of this second injection was 1 μc per g in two animals, and 1 μc per 4 g in the second pair. I each experiment the incidence of labelled and unlabelled small lymphocytes was recorded in total of 4000 lymphocytes, 1000 of these cells being counted in each of four smears.

Group 3 experiments: 6 experiments were made in this group. Guinea-pigs were injected intra-venously with ^{3}H thymidine (specific activity 3.0 per mM, dosage 1 μc per g body weigh) and the bone marrow removed from the long bones of all four limbs within 30 minutes of the injection (this interval precludes the labelling of daughter cells, including small lymphocytes). The marrow cells were suspended and washed in serum obtained from litter-mate. After adjusting the volume to 2 ml, the suspension of labelled marrow cells was injected into the peritoneal cavity of recipient who, 24 hours previously had been given 1000 rads whole body irradiation. The marrow suspension remained in the animal for 7½ hours, and the guinea-pig was then anaesthetised, its peritoneal cavity opened and the cell suspension aspirated with pipette. Smears were prepared from this marrow suspension and were processed for autoradiography.

Irradiation of Animals

) Shielding experiments: The animal was anaesthetised with intraperitoneal sodium pentobarbitone and inclined head downwards, supported by its feet, on sloping wooden platform (30° to the horizontal). I this way the intestinal tract, and its related lymphoid tissue were displaced into the upper part of the abdominal cavity beneath the diaphragm and well within the field of irradiation. The lower limbs were bandaged carefully together as far as the groin, and were then covered with 4 mm lead-shield, which extended to the root of the limb. By use of the bandage gut was prevented from lying beneath the proximal edge of the lead shield. Whilst the hind-limbs were shielded, the remainder of the body was irradiated. Irradiation was given from an -ray 220 kv 15 ma pparatus, filters 1 mm Cu and 1 mm Al, H.V.T. 1.5, SSD 40 cm. At the midpoint of the irradiation time, each animal was turned through 180° so that its opposite flank was nearest to the irradiation source, and the irradiation was then completed. During irradiation, measurement was made of the 'scatter' beneath the lead-shield the level of the mid-shaf of the femur using Baldwin-Farmer Electrometer Unit.

b) Whole-body irradiation. In Group 3 experiments, animals used as hosts for intraperitoneal injection of bone marrow were given supra-lethal irradiation from ^{60}Co unit before receiving the marrow. The conditions and method of whole-body irradiation ha been previously described (8).

Lethal dose of irradiation for guinea-pigs of Dunkin-Hartley strain. Recent studies (9) have shown tha 720 rads is lethal dose of irradiation to 100% of guinea-pigs of the Dunkin-Hartley strain within 15 days of irradiation.

Autoradiography: The method used was based upon the stripping-film technique described by PELC (17) and full details of the handling of bone marrow smears have been described elsewhere (7). The smears were stained through the stripping film with 0.1 solution of New Methylene Blue. Lymphocytes were not recorded as labelled unless the number of grains overlying the nucleus exceeded alue of 5.

Quantitative technique to estimate marrow cellularity: The cellularity of shielded and irradiated marrow was estimated quantitatively using bone marrow suspension technique described by YOFFEY (23) and modified by HARRIS (6). By pplying the percen-

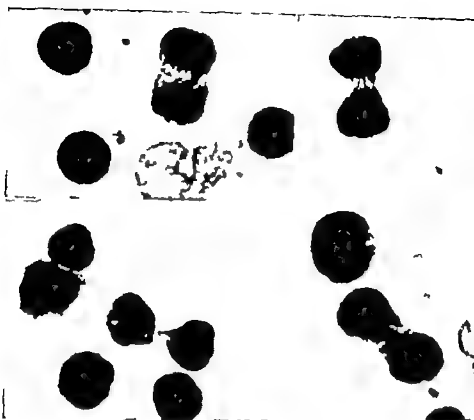


Fig 1a—d Telophase mitoses in smears of guinea-pig bone marrow showing formation of small lymphocytes. MacNeal Tetrachrome. 1350.

tage obtained in differential counts of cells in marrow smears to the bsolute number of nucleated cells estimated by the quantitati technique the absolute number of lymphocytes per unit olume of shielded or irradiated marrow was obtained.

Results

Telophase mitoses forming small and medium-sized lymphocytes in guinea pig bone marrow Examples of mitotic figures in telophase in guinea pig bone marrow are shown in fig 1 These cells were recorded in smears prepared from bone marrow recovering from sub-lethal irradiation at a stage when it contains large numbers of small lymphocytes Particularly notable features are the small size of the daughter nuclei and the paucity of cytoplasm associated with them. These cells are very characteristic and cannot be confused with any other type of marrow cell. In fig 2 daughter cells are

illustrated immediately after their separation in telophase They have all the features of small lymphocytes except that their nuclear chromatin still shows some evidence of condensation.



Fig 2. Small lymphocyte daughter-cells in bone marrow smear shortly after separation of telophase MacNeal' Tetrachrome. 1350

Total nucleated cell levels in shielded and irradiated bone marrow The total nucleated cell counts in the shielded and non shielded marrows after three different doses of irradiation are shown in table 1 These doses are either equal to the LD 100 or are even higher being in the supra lethal range.

There are two salient findings in these experiments. Firstly in the *marrow exposed to irradiation* in the lethal range (720 rads) the depression of total cellularity is very marked, the levels actually falling to only 8% of the normal value. After higher doses of irradiation the marrow appeared to be even more depleted of cells. Thus after 1000 rads, and following two divided doses of 1000 and 500 rads, the cell numbers were so small and the bone marrow was so gelatinous and haemorrhagic that the cells could not be effectively suspended and attempts to estimate the total levels of nucleated cells had to be abandoned. Secondly the *shielded bone marrow* also showed a noteworthy reduction of its total nucleated cells. Seven days after 720 rads irradiation, the cellularity of the shielded marrow was reduced to just over 60% of the control value. It was just less than 60 / after 1000 rads, and after 1500 rads (given as two divided doses) the total number of nucleated cells was even further reduced being only 35% of normal

Dose of irradiation received by the shielded marrow The dose of scatter irradiation measured at mid-shaft of the lead-shielded femur was 12 rads in those animals which had received 720 rads to the rest of the body and 17 rads in those which had been given 1000 rads.

Table I

Total nucleated cells in shielded and non-shielded guinea-pig bone marrow 7 days after irradiation and shielding

Dose of irradiation shielding of hind-limbs	Total nucleated cells per cu.mm marrow (mean ± standard error)			
	Femoral marrow (shielded)	As % of control level	Humeral marrow (non-shielded)	As % of control level
Controls	1,814,000 ± 86,000		1,814 000 ± 86,000	
720 rads	1 124 000 ± 87,000	62*	154,000 ± 19,000	8
1000 rads	1,074,000 ± 101 000	59*	Too low for accurate estimation.	
1000 – 500 rads	626,000 ± 84,000	34	Too low for accurate estimation.	

Table II

Lymphocyte content of shielded and non-shielded guinea-pig bone marrow and blood lymphocyte levels 7 days after irradiation and shielding

Dose of irradiation, shielding of hind limbs	Medium small lymphocytes in shielded (femoral) bone marrow (cells per cu.mm marrow. Mean ± standard error)	[illegible] of control level	Medium small lymphocytes in non-shielded (humeral) bone marrow (cells per cu.mm marrow. Mean standard error)	[illegible] of control level	Blood lymphocytes (per cu.mm blood. Mean ± standard error)	Lymphocyte levels as % of control level
Controls	361,000 ± 33,000		361,000 ± 33,000		3,680 ± 130	
720 rads	280 000 ± 19 000	78*	18,000 ± 2,000	5	880 ± 90	24
1000 rads	176,000 ± 10,000	49*	too low for accurate estimation		390 ± 30	11
1000 – 500 rads	122,000 ± 3,000	34	too low for accurate estimation		190 ± 30	5

Lymphocyte levels in shielded and irradiated marrows and blood lymphocyte levels Table II shows the number of small and medium lymphocytes per unit volume of bone marrow in the irradiated and shielded limbs after doses in the lethal and supra lethal range Seven days after 720 rads irradiation the lymphocyte levels in the irradiated marrow are only 5 of the control value After 1000 and 1500 rads the levels appear to be even lower but they could not be

accurately measured due to the difficulty in establishing the total nucleated cell levels. In contrast with the irradiated marrow that in the shielded limbs still contains a considerable number of lymphocytes. In animals receiving 720 rads irradiation, the lymphocyte levels in the shielded marrow are approximately 80 % of normal. After larger doses of irradiation there is a greater reduction in the lymphocyte population of shielded marrow Thus, after 1000 rads the lymphocyte levels, although still considerable, fall to approximately 50 % of the normal value, and after 1500 rads their level is approximately 35 % of normal.

Blood lymphocyte levels confirm the marked sensitivity of lymphoid tissue to irradiation. After 720 rads, with shielding of the hind limbs, the blood lymphocyte levels are only 24% of the control level on the 7th day after irradiation. After 1000 rads they are only 11% of normal, and after 1500 rads they are 5 % of normal The absolute levels of blood lymphocytes following lethal and supralethal doses of irradiation are shown in table II.

The presence of newly-formed lymphocytes in shielded bone marrow The percentages of newly formed small lymphocytes in shielded marrow are shown in table III Precursor cells in the shielded marrow were labelled with ^{3}H thymidine and after an interval of 48 h the proportion of labelled small lymphocytes which appeared in this marrow was recorded. Examples of labelled cells are illustrated in fig 3 These lymphocytes appear to be formed from labelled precursors in the shielded marrow This marrow is actively proliferating as shown by the high incidence of labelled mitoses in smears (fig 4) After a single dose of ^{3}H-thymidine the proportion of labelled small lymphocytes which appear in shielded marrow varies between 17—52% The figure of 17% is rather low and may be associated with the low dosage rate of thymidine (1 μc per 3 g body weight) given in this particular experiment. In animals given 1 μc per g body weight, the range of labelled small lymphocytes is 31—52 % with a mean of 36 %. The proportion of labelled cells is greater in those animals which were given a second dose of label, 24 h after the first injection. In these, a mean of 45 % (range 33—54 %) of the small lymphocyte population present in the shielded bone marrow after lethal irradiation of the rest of the body appear to be newly formed.

Histological appearance of irradiated lymphoid tissues in animals with shielded bone marrow Sections of irradiated thymus, spleen and

Table III

The incidence of newly-formed small lymphocytes in shielded bone marrow of lethally irradiated guinea-pigs.

Expt. No.	Time after lethal irradiation when shielded marrow examined, days	No. of injections of ³H-thymidine	Duration of exposure to ³H-thymidine hours	Dosage rate ³H-thymidine 1st injection, μc/ body wt	Dosage rate ³H-thymidine 2nd injection, μc/ body wt	Percentage of small lymphocytes, labelled with ³H-thymidine	
1	4	1	48	1 μc/1 g	—	31	
2	3	1	48	1 μc/3 g	—	17	
3	7	1	48	1 μc/1 g	—	24	Mean = 31
4	4	1	48	1 μc/1 g	—	52**	
5	4	2	48	1 μc/1 g	1 μc/1 g	35*	
6	4	2	48	1 μc/1 g	1 μc/1 g	54	Mean = 45
7	4	2	48	1 μc/1 g	1 μc/4 g	48*	
8	4	2	48	1 μc/1 g	1 μc/4 g	44	

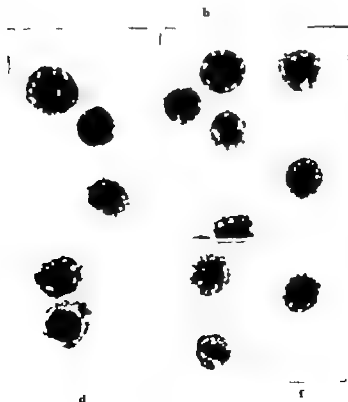


Fig. 3a–f Small lymphocytes labelled with ³H-thymidine in shielded bone marrow of lethally irradiated guinea-pigs. New Methylene Blue × 1370.

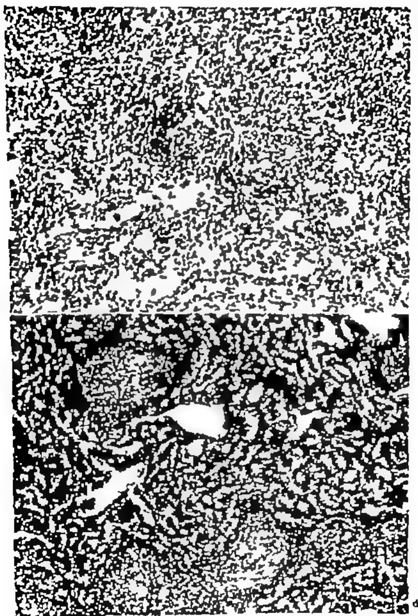


Fig 4a—e. Mitotic figures in telophase labelled with ³H-thymidine in shielded bone marrow of lethally irradiated guinea-pigs. New Methylene Blue. [illegible]

mesenteric lymph node in guinea pigs with shielded bone marrow are shown in fig 5 After either 720 or 1000 rads the findings are similar There is very considerable depletion of lymphocytes in the various lymphoid tissues. The thymic lobules are small and the cortex is so deficient in small thymocytes that the lobules show an inversion pattern (21) in which the cortex has a pale, epithelial like appearance. In the spleen the lymphoid follicles all have a similar appearance. There is a thin outer zone of moderate cellularity but the bulk of the follicle appears pale due to the paucity of lymphocytes. In the lymph-node, the follicles are uniformly small and poorly formed and show no evidence of any significant lymphocytopoiesis. The diffuse lymphoid tissue of the cortex is thin and poorly developed



Fig 5 —A Th mus. 7 days after 720 rads irradiation showing inverse pattern in lobules. 134 b Th m.s 7 days after 1000 rads irradiation. 70 L ymph-node 7



days after 720 rads irradiation. 134 d) Spleen, 7 days after 720 rads irradiation. 50.
Sections stained Haematoxylin and Eosin.

Formation of small lymphocytes in isolated bone marrow cultured in vivo. Precursor cells in bone marrow were labelled by injecting ^{3}H thymidine into a guinea pig. This marrow was then removed for culturing and was extracted before daughter cells could have entered it from the donor's own lymphoid tissues. Before culture the labelled precursor cells were washed in homologous serum. They were cultured for only 7½ h in the peritoneal cavity of a previously irradiated host. Since the labelling of large numbers of cells was not anticipated no attempt was made to accurately measure the absolute number of labelled cells. In the smears which were studied, the incidence of labelling in small lymphocytes extended over the range 2.6—3.5 1000 small lymphocytes being counted in each experiment and using two smears for the purpose. Examples of

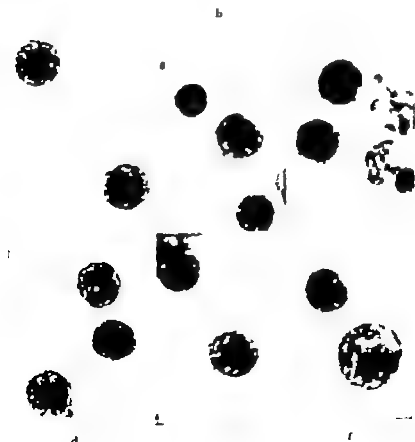
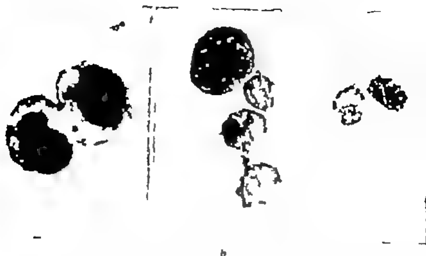


Fig. 6a—f. Small lymphocytes labelled with ^{3}H-thymidine in smears of isolated, cultured guinea-pig bone marrow. New Methylene Blue. × 1350.



b

Fig 7a—c Telophase mitoses labelled with ^{3}H-thymidine in smears of isolated, cultured guinea-pig bone marrow The mitoses in figs. 7b and 7 are forming small and medium-sized lymphocytes. New Methylene Blue 1330.

labelled small lymphocytes in smears of cultured marrow are shown in fig 6 Labelled mitoses in telophase indicating active proliferation in the cultured marrow are shown in fig 7

Discussion

The formation of lymphocytes in guinea-pig bone marrow The present studies are complementary and provide evidence for the formation of small lymphocytes in situ in bone marrow Moreover the data suggest that the numbers formed in this tissue may be considerable.

The shielding experiments show that despite lethal or supra lethal irradiation of the rest of the body considerable numbers of lymphocytes still remain in the shielded marrow The levels are almost 80% of normal after 720 rads, approximately 50% after 1000 rads, and about 33 % after 1500 rads. It is extremely unlikely that the large population of lymphocytes in the shielded marrow results from lymphocyte migration into it after irradiation. With the exception of lymphocytes in the shielded marrow the levels of these cells in the remainder of the body would have been profoundly affected in two ways (11) by the dose of irradiation used firstly by suppression of lymphocytopoiesis, and secondly by direct destruction of small lymphocytes which are very sensitive to x rays (20) From the data of HEWITT AND WILSON (10) a dose of 720 rads destroys or

damages approximately 98 % of mammalian cells with reproductive capacity 1000 rads affects 99 6% and 1500 rads will affect 99 98 The efficacy of irradiation given in the present studies is shown by the marked depression of lymphocyte levels in the irradiated marrow (only 5% of the control level after 720 rads) and by the marked lymphopoenia in the peripheral blood (table II)

How may the population of lymphocytes which remain in the shielded marrow be interpreted? Since many of these cells have a long life-span (16) it might be assumed that the lymphocytes were present in the marrow before the irradiation and that they migrated there from conventional lymphoid tissues This supposition is incorrect. The use of a DNA label in the present studies shows that a substantial proportion of lymphocytes in the shielded marrow are *newly formed* after the animal was severely irradiated Continuous infusion of ^{3}H thymidine would have achieved maximum labelling of daughter cells but even after two injections of the label, approximately 45 of the lymphocytes appear to be recently formed in the marrow In the irradiated animals lymphoid tissues, including thymus, spleen and mesenteric lymph node, were severely damaged There is, therefore no evidence that the large number of young lymphocytes in the shielded marrow were formed in extraneous sites and then migrated into the marrow Even if a small number did migrate there from other lymphoid tissues, it is most probable that their precursors originated in the shielded marrow and in fact seeded the irradiated lymphoid tissues. The incidence of labelled small lymphocytes in shielded marrow in the present studies is comparable with that in bone marrow recovering from sub-lethal irradiation (7) in which 46 % of small lymphocytes were labelled 48 h after the injection of ^{3}H thymidine The figure is also comparable with that found for small lymphocytes in bone marrow of normal guinea pigs (15)

The detection of young newly formed lymphocytes in shielded bone marrow after lethal or supra-lethal irradiation of the rest of the body prompted the final investigation in the present studies. Bone marrow from donor animals, in which only proliferating cells had been labelled with ^{3}H thymidine, was cultured in vivo in the peritoneal cavity of a supra lethally irradiated host. Small percentages (up to 3.5 of labelled small lymphocytes were found in the marrow suspension after culture for 7$^1/_2$ hours Since some of the labelled cells may have migrated away from the peritoneal cavity

during the period of culture, the percentage of labelled lymphocytes found in the smears may be an under-estimate The most reasonable interpretation is that the labelled small lymphocytes were formed in the isolated bone marrow For two reasons, it is highly improbable that the labelled cells were of host origin. Firstly the dose of irradiation given to the host before injection of labelled marrow would have profoundly depressed its own lymphocytopoiesis. Moreover for the host's haemopoietic cells to acquire the label it would have to be released from the labelled marrow cells into the general circulation before being re utilized by them The amount of ^{3}H thymidine so released would be greatly diluted in the circulation. It is doubtful whether it would even be detectable in cells of the host. Using peritoneal diffusion chambers in guinea pigs with normally functioning lymphoid tissues, OSMOND AND EVERETT (15) cultured bone marrow previously labelled with ^{3}H thymidine. They found that 12% of marrow lymphocytes were labelled after 26 hours culture. However a small number of lymphocytes in their marrow were already labelled by the time the culture was commenced, and also they could not exclude that labelled DNA was being re-utilized by cells in the confined space of the diffusion chamber

The present studies indicate that small lymphocytes are produced in considerable numbers in shielded bone marrow and that they are also formed in normal marrow These findings are supported by recent experiments (13 15) in normal animals in which marrow lymphocytes have been studied by temporarily occluding the circulation to the bone marrow in a limb whilst ^{3}H thymidine is made available to the remainder of the haemopoietic tissues. Experiments by other workers also support lymphocyte production in bone marrow In the rat, BIERRING (1 2) found that bone marrow lymphocyte levels were not affected by thymectomy together with removal of 70% of lymph-node tissue. Transfusion experiments (4 18) in which bone marrow cells of donor animals re-populate lymphoid tissues as well as the recipient's bone marrow favour the presence of lymphocytopoietic cells in bone marrow OSMOND AND EVERETT (15) observe that although their studies support lymphocyte production in bone marrow there appears to be a paucity of recognizable lymphocytopoietic cells in guinea-pig marrow It must be stressed that this paucity does not exist in the irradiated guinea pig Many lymphocytopoietic cells are easily recognizable in mar

row regenerating after irradiation (7 8) The present experiments do not establish whether the lymphocyte precursors originate in lymphoid tissues and enter the blood stream to reach the marrow where they divide to form small lymphocytes, or whether they originate in foetal marrow and persist in the marrow post natally Also they do not indicate whether small lymphocytes of bone marrow are similar to those formed in typical lymphoid tissues.

The decreased cellularity of shielded bone marrow An unexpected finding in the present experiments is that the total nucleated cells in the shielded marrow are considerably reduced. This occurs despite efficient shielding of the limbs The reduction is too great to be explained by haemopoietic depression following the small dose of scatter irradiation which the shielded marrow received. It seems probable that this marrow is subjected to excessive functional demands which are usually discharged by a much larger volume of marrow In normal marrow there is a large granulocyte reserve but this may be almost exhausted in the shielded marrow if it attempts to satisfy the requirements of the whole body This would explain a decrease in the cellularity of the shielded marrow An additional mechanism may also contribute to the reduction in cells. Haemopoietic cells may have been exported from the shielded marrow to seed haemopoietic tissues in the rest of the body since it is known that shielding of only a small volume of bone marrow in irradiated animals facilitates recovery from irradiation (12 19) Further studies will be reported in which a detailed quantitative analysis is made of the various cell populations in shielded and irradiated marrows, at close intervals following exposure of the rest of the body to a lethal dose of irradiation

The reduction of lymphocytes in shielded bone marrow The depletion of lymphocytes is too great to be due to the small dose of scatter irradiation received by the shielded bone marrow This marrow may discharge its own lymphocytes into the blood stream in an attempt to compensate for the profound deficiency of these cells in the rest of the body A further explanation is indicated by the work of Keiser et al. (15) whose studies suggest that small lymphocytes in the bone marrow arise from two sources, some being formed from local precursors already in the marrow and others migrating into it via the blood stream In the present experiments the lymphoid tissues were very severely damaged by irradiation and this would effectively suppress one source of lymphocytes. Thus, the fall in

lymphocyte content of the shielded marrow m[illegible] result from loss of those lymphocytes which arise extrinsically [illegible] those remaining may represent cells which arise from local prec[illegible]ors. The decrease in lymphocyte levels in the shielded marrow m[illegible] also be related to irradiation damage of the thymus. Removal [illegible] the thymus (14) causes an atrophy of lymphoid organs. This is [illegible] to the deficiency of a lymphocytosis stimulating factor (LSF [illegible] produced by the thymus, which promotes the formation of sma[illegible]mphocytes. If the severely irradiated thymus produces very litt[illegible]SF then this ma[illegible] lead to a reduction in marrow lymphocytopo[illegible] and consequently a reduction in the numbers of bone marrow ly[illegible]ocytes. However the experiments of BIERRING (1 2) suggest t[illegible] marrow lymphocyte levels may be independant of other lymp[illegible]d tissues, including the thymus.

Acknowledgements. W[illegible] wish to thank Mr DAVID [illegible], B. Sc., Sheffield Regional Medical Physics Department, for advice and assistance with guinea pig irradiation, also Miss M. Toon for skilled technical assistance and photography

Summary

Evidence from [illegible] variety of experiments indicates that lymphocytes are produced in guinea-pig bone marrow Under certain conditions the numbers produced [illegible]ppear to be considerable. During recovery from irradiation there are numerous telophases in marrow smears which [illegible]ppear to be forming small lymphocytes. Moreover many lymphocytes are still found in shielded bone marrow after the rest of the body including the conventional lymphoid tissues, has received lethal or supra-lethal irradiation Almost 50% of these cells are formed after the irradiation Finally when bone marrow is cultured in the peritoneal cavity of [illegible] host whose own lymphocyte production has been suppressed by irradiation, small percentages of newly-formed small lymphocytes are found in this marrow

Résumé

Différentes expériences mettent en évidence que des lymphocytes sont formés dans la moelle du cobaye Dans certaines conditions, leur nombre semble être considérable. Durant la régénération qui suit une irradiation, les frottis de la moelle montrent de nombreuses télophases qui semblent donner naissance à de petits lymphocytes. En plus, on trouve de nombreux lymphocytes dans la moelle osseuse qui [illegible] été protégée des rayons après que le reste de l'organisme, y compris les tissus lymphatiques conventionnels, ait reçu une irradiation léthale ou supraléthale. A peu près 50% des cellules son[illegible] formées [illegible]près l'irradiation. Enfin, si la moelle osseuse est cultivée dans la cavité péritonéale d'un hôte dont la production de lymphocytes [illegible] été supprimée par irradiation, on y trouv[illegible] un petit pourcentage de lymphocytes jeunes.

Zusammenfassung

Aus verschiedenen U[illegible]tersuchungen geht hervor dass im Knochenmark des Meer[illegible]sch[illegible] einchens Lymphocyten gebildet werden. Unter gewissen Bedingungen scheinen diese Prod[illegible]ktion beträchtlich zu sein. In der Erholungsphase nach Bestrahlung finden sich im Knochenmark Telophasen, [illegible]us denen kleine Lymphocyten hervorzugehen scheinen. Überdies werden im abgeschirmten Knochenmark noch zahlreiche Lymphocyten gefunden, nachdem der übrige Körper einschließlich des üblichen lymphatischen Gewebes, letalen oder supraletalen Strahlendosen ausgesetzt worden war. Nahezu 50% dieser Zellen werden nach der Bestrahlung gebildet. Wird schließlich Knochenmark in der Peritonealhöhle eines Tieres kultiviert, dessen eigene Lymphocytenproduktion durch Bestrahlung unterdrückt wurde, so findet sich darin noch ein kleiner Prozentsatz neugebildeter kleiner Lymphocyten.

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Index autorum ad Vol. 33

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